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RESULTS OF VACCINATION AGAINST INFLUENZA DURING THE EPIDEMIC OF 1947

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INTRODUCTION

THE influenza epidemics of 1943 and 1945 respectively provided the opportunity for several groups of investigators[1-5] to evaluate the use of combined influenza A and B virus vaccine as a means of protection against this disease. These studies yielded highly significant results in favor of the prophylactic value of vaccination even when the vaccine was given as long as a year preceding an outbreak.[5] Although these investigations established without doubt the importance of vaccination as a control procedure during the 1943 and 1945 epidemics, further evaluation during subsequent outbreaks in civilian groups seemed warranted. Studies on the periodicity of epidemic influenza[6] indicated that sometime during the fall and winter of 1946-1947 an outbreak would occur. In the fall of 1946, therefore, a study was organized at the University of Chicago to test further the usefulness of vaccination for protection against influenza.

METHODS

Population.—Two thousand and twenty students (men and women) living in twelve University houses were employed in the epidemiologic study. Seven hundred ninety volunteers were vaccinated and 1,230 served as controls. The vaccination program was carried out in the dormitories over a period from Nov. 6 to Dec. 6, 1946. Not more than 50 per cent of the population in a given house was vaccinated. A record was kept of each student in the test and control groups in the Student Health Clinic and all respiratory illnesses were recorded. The epidemiologic data were derived from patients reporting to the clinic for care. In order to obtain crude data on the over-all incidence of influenza, a questionnaire was sent to all the students in the test and control groups on April 6 inquiring whether they had had an illness with symptoms resembling influenza during the previous six weeks which included the epidemic period.

In addition to those in the dormitories, several hundred students living in private homes, fraternity houses, and so forth, were also vaccinated in the

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clinic. These, along with several hundred unvaccinated students not included in the epidemiologic group, were cared for in the clinic or hospital during the epidemic period. Sera from both acute and convalescent phases also were obtained from many in these groups for serologic diagnosis of influenza.

Vaccines.—Three different commercially prepared inactivated virus vaccines* having an A component of 25 per cent PR-8 and 25 per cent Weiss strains and a B component of 50 per cent Lee strain were employed. The number receiving each brand of vaccine was approximately the same. Each student in the test group was given 1 c.c. of vaccine subcutaneously in the deltoid region of the right or left arm. Skin tests to determine sensitivity to the vaccines were not performed prior to vaccination. The volunteers were questioned about sensitivity to eggs, fowl feathers, or to previous vaccinations. Those giving positive histories were placed in the control group. Only minor local reactions and an occasional moderately severe generalized reaction to the vaccines consisting of fever and malaise were encountered.

RESULTS

Immune Response to Vaccination.—The method employed for antibody determination was Salk's[7] modification of the Hirst[8] chicken red cell agglutination inhibition technique. The immune response to vaccination (Table I) was satisfactory, as shown by a comparison of the antibody titers against the virus strains in the vaccines in sera collected before and from two to three weeks after vaccination. There was no significant difference in the immune response to the three vaccine preparations.

TABLE I. MEAN INFLUENZA ANTIBODY TITERS BEFORE AND TWO TO THREE WEEKS AFTER VACCINATION SUBCUTANEOUSLY WITH 1 C.C. OF COMBINED A AND B INFLUENZA VIRUS VACCINE.

VACCINE PREPARATION	VIRUS STRAINS	PAIRS OF SERA	ANTIBODY TITERS	
			PREVACC.	POSTVACC.
Parke, Davis*	PR-8	94	374	1944
	Weiss	95	224	1075
	Lee	111	156	512
Eli Lilly†	PR-8	97	294	1075
	Weiss	94	137	914
	Lee	115	114	433
Lederle‡	PR-8	90	255	806
	Weiss	95	182	530
	Lee	93	104	465

*Calcium phosphate adsorption inactivated by ultraviolet light.
†Chicken red cell eluate inactivated by formalin.
‡High-speed centrifugation inactivated by formalin.

The 1947 Epidemic.—Late in January, 1947, outbreaks of influenza, found to be due to influenza virus type A, were reported in Army population groups in California, Colorado, and New Jersey.[9] Subsequently, the military personnel of other Army installations near Chicago were attacked. During this period when the Army was having its main incidence of influenza, the civilian popula-

*Kindly supplied by Lederle Laboratories, Inc., New York, N. Y., Eli Lilly & Company, Indianapolis, Ind., and Parke, Davis & Company, Detroit, Mich.

tion appeared to be relatively free from it. The peak of the epidemic among civilians occurred during the latter part of February and the early part of March.[10-15]

A sharp increase in the incidence of acute respiratory disease occurred among the student population at the University of Chicago during the first week of March, 1947. The majority presented symptoms characteristic of mild influenza with sudden onset, malaise, frontal headache, back pain and generalized muscle pains, and nonproductive cough. Only a few complained of sore throat

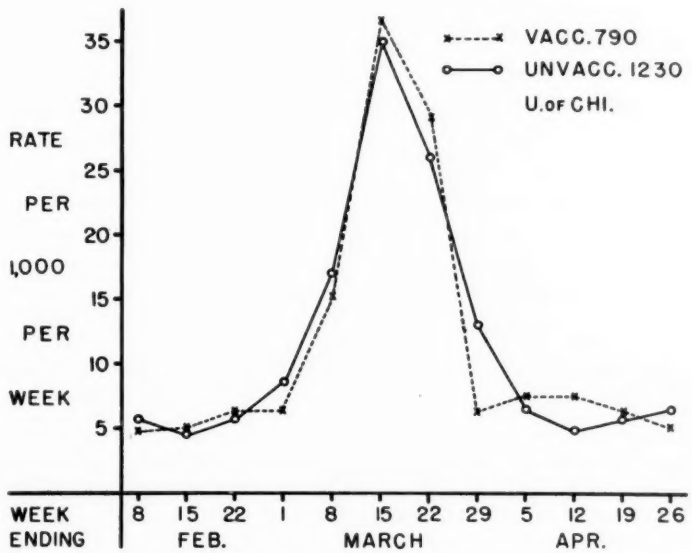

Fig. 1.—Incidence of acute respiratory disease among vaccinated and unvaccinated students reporting to the clinic for care during the epidemic period of influenza, 1947.

or stuffy nose or suffered from nausea or vomiting. The epidemic was of short duration, reaching its peak March 15 and lasting a period of about four weeks (Fig. 1). Before and after the period of increased incidence, the weekly rate for respiratory disease was low and was essentially the same in the vaccinated and unvaccinated groups (Fig. 1).

Incidence.—The data shown in Table II are based on the number of students coming to the clinic and admitted to the hospital for care for acute respiratory disease. The criterion for admission to the hospital was generally a fever of 101° F. or above with corresponding constitutional symptoms and cough. During the period of increased incidence of acute respiratory disease from March 2 to April 5, 1947, the attack rate was the same in the vaccinated and unvaccinated groups, being 9.5 per cent. Two and five-tenths per cent of the vaccinated and 2.26 per cent of the unvaccinated students were considered sufficiently ill to be admitted to the hospital (Table II). Five hundred seventy-six students from the control group and 606 from the test group returned the respiratory disease questionnaire. Forty-one and eight-tenths per cent of those

answering from the control group and 46.5 per cent of the test group described symptoms of upper respiratory tract infection. The great majority dated their illness during the period of increased incidence as noted from the clinic records.

TABLE II. INCIDENCE OF ACUTE RESPIRATORY DISEASE DURING EPIDEMIC PERIOD FROM MARCH 2 TO APRIL 5, 1947

GROUP TREATED IN	VACCINATED (790)		UNVACCINATED (1230)	
	NUMBER OF CASES	INCIDENCE	NUMBER OF CASES	INCIDENCE
Clinic	55	7.00	88	7.14
Hospital	20	2.50	29	2.36
Total	75	9.50	117	9.50

Serologic Diagnosis of Influenza A.—From Feb. 8 to April 26, 1947, acute and convalescent phase blood was collected from 192 unvaccinated individuals showing respiratory illnesses suggestive of influenza. These were tested for antibody rise to the PR-8 and Lee strains of influenza virus. No significant rise was noted against the Lee strain in any of the specimens of convalescent sera. On the other hand, as is shown in Table III, 40 per cent of the blood specimens during the first three weeks of March showed a fourfold or greater rise in titer to the PR-8 strain of influenza A virus. Only two of the twenty-nine blood specimens collected before and after the period of increased incidence of illness, however, showed significant rises in titers to PR-8.

TABLE III. ANTIBODY RESPONSE TO TYPE A (PR-8 STRAIN) INFLUENZA VIRUS IN UNVACCINATED INDIVIDUALS SHOWING SYMPTOMS OF INFLUENZA IN 1947

PERIOD OF TIME	FEBRUARY 8-28	MARCH 2-8	MARCH 9-15	MARCH 10-22	MARCH 23-29	MARCH-APRIL 30-5	APRIL 6-26
Per cent*	16	40	41	39	28	15	13
Convalescent sera*	1	16	16	16	9	2	1
Pairs of sera	6.2	40.0	39.0	41.0	32.0	13.4	7.7

*Convalescent sera showing a fourfold or greater rise in titers.

Etiological Diagnosis of Influenza.—Throat washings were collected from thirty-two hospitalized patients immediately after admission by having them gargle with 10 to 20 c.c. of nutrient broth. The throat washings were then frozen and kept in the dry ice chest until ready for study. Two strains of influenza virus, J-16 and L-32, were isolated by direct inoculation of 0.2 c.c. of the unfiltered, undiluted throat washings into the allantoic cavities of 10-day-old chick embryos on the third egg passage. After the first passage, 0.1 c.c. of a 10^{-2} dilution of the allantoic fluid was employed. Penicillin and streptomycin (100 units per 1.0 c.c. of undiluted washings) were added one-half hour before the initial inoculation was made and occasionally before subsequent passages. The eggs were incubated for forty-eight hours at 99 to 100° F. All samples of throat washings were subjected to at least four egg passages before they were discarded.

Antigenic Relationship Between Influenza A Virus PR-8 and the 1947 Strains.—Sixty-eight pairs of sera from unvaccinated individuals, collected be-

fore and after the infections, again were tested for antibody levels against the PR-8 as well as against the recently isolated J-16 and L-32 strains (Table IV). Thirty-seven of the convalescent sera showing a fourfold or greater rise in antibody titers against PR-8 also showed significant rise in titers to strains J-16 and L-32. Likewise, as shown in Table IV, thirty-one pairs of sera showing no significant rise in titer to PR-8 also showed no rise to the 1947 strains.

TABLE IV. INFLUENZA ANTIBODY TITERS FOR THE PR-8, J-16, AND L-32 VIRUS STRAINS IN SERA FOLLOWING VACCINATION WITH COMBINED A AND B VACCINE CONTAINING PR-8 STRAIN AND INFECTION IN UNVACCINATED INDIVIDUALS

	NUMBER OF PAIRS OF SERA TESTED		
	POSTVACCINATION	CONVALESCENT	
	60	37*	31†
VIRUS STRAINS	MEAN FOLD INCREASE		
PR-8	7.1	5.6	1.6
J-16	1.3	5.3	0.7
L-32	1.8	5.1	0.7

*Showed fourfold or greater increase in titer at the time of original testing for PR-8 influenza antibodies.

†Showed threefold or less rise in titers to PR-8 influenza antibodies at time of original testing.

Sixty pairs of sera collected at the time of vaccination also were retested for antibody response against PR-8 and the 1947 viruses (Table IV). Whereas the postvaccination sera showed an average increase in antibody to PR-8 of over sevenfold, no significant rise was noted to the J-16 and L-32 strains. These data show that there was no close antigenic relationship between influenza viruses causing the 1947 outbreak and the influenza A component in the vaccine. Yet the increase in antibody titers in the convalescent sera to the J-16 and L-32 and also to the PR-8 strain establishes the first two as type A influenza viruses.

Serologic Studies on Vaccinated Individuals.—Acute and convalescent phase sera were obtained from 46 vaccinated individuals in the test group who came to the clinic or were hospitalized for care between March 2 and April 5. Twenty-three pairs (50 per cent) showed a fourfold or greater rise to the J-16 strain of influenza virus. The majority of those showing significant rises showed less rise in antibody titer to the PR-8 strain, in all probability because of the initially high antibody level in the acute phase sera (Table V).

Pre- and postvaccination blood specimens had been obtained from 25 of the vaccinated individuals from whom acute and convalescent sera were collected at the time of illness. Fourteen (56 per cent) showed significant rises in titer in the convalescent sera to the J-16 virus but no rise in titer to this strain in the postvaccination sera (Table V). On the other hand, vaccination provoked significant rises in titer to the PR-8 strain, and the antibody levels to PR-8 were only slightly lower in the acute phase sera collected at the time of illness than those present in the postvaccination samples (Table V). The low antibody titers to the closely related J-16 and L-32 influenza virus strains and the relatively high antibody levels to PR-8 in the acute sera of vaccinated individuals together with the significant rise in titers following infection establish the former strains as the etiological agents of the 1947 influenza outbreak.

TABLE V. COMPARISON OF INFLUENZA ANTIBODY TITERS FOR THE PR-8 AND J-16 STRAINS IN SERA FOLLOWING VACCINATION WITH COMBINED A AND B VACCINE CONTAINING PR-8 STRAIN AND INFECTION IN VACCINATED INDIVIDUALS

		VACCINATION SERA			INFECTION SERA		
CASE	VIRUS STRAIN	PRE-	POST-	FOLD RISE	ACUTE	CONVAL.	FOLD RISE
C-99	PR-8	32	1024	10	512	4096	6
	J-16	32	32	0	16	128	6
764-D	PR-8	64	2048	10	512	1024	2
	J-16	32	64	2	32	256	6
161-Z	PR-8	64	1024	8	1024	1024	0
	J-16	32	32	0	32	128	4
197-Z	PR-8	64	8192	14	8192	8192	0
	J-16	16	32	2	64	512	6
125-B	PR-8	128	1024	6	512	1024	2
	J-16	16	16	0	16	128	6
299-B	PR-8	64	2048	10	2048	4096	2
	J-16	32	32	0	32	128	4
586-B	PR-8	4096	4096	0	2048	8192	4
	J-16	32	64	2	32	512	8
594-B	PR-8	128	2048	8	1024	4096	4
	J-16	16	16	0	16	128	6
266-B	PR-8	2048	4096	2	2048	4096	2
	J-16	64	64	0	64	512	6
1040-V	PR-8	128	4096	10	512	1024	2
	J-16	32	64	2	32	1024	10
1027-V	PR-8	64	2048	10	512	1024	2
	J-16	32	64	2	32	256	6
105-V	PR-8	256	512	2	1024	2048	2
	J-16	64	64	0	16	256	8
1057-V	PR-8	256	512	2	256	512	2
	J-16	64	64	0	128	512	4
187-V	PR-8	512	2048	4	4096	4096	0
	J-16	32	32	0	32	128	4

DISCUSSION

A year previous to the 1943 influenza A epidemic, Salk and associates[5] inoculated a large number of subjects in a state institution with a combined influenza A and B vaccine similar to one of the preparations employed in this study. The response to the vaccine was good and sera from vaccinated individuals even after a year showed antibody levels several times higher than the prevaccination titers. Proof of the immune state of the vaccinated group after a year compared with the control group was shown in the markedly lower incidence of infection in the former—1.9 per cent compared with 12.4 per cent in the latter. The larger study for the evaluation of influenza vaccines conducted by members and associates of the Commission on Influenza, Army Epidemiological Board, during the 1943 epidemic likewise yielded results significantly in favor of vaccination.[1, 2] The incidence of infection was 2.2 per cent in the test group and 7.1 per cent among the controls. In this study the inoculations were given immediately before and up to twelve weeks previous to the outbreak of the epidemic. Among the group vaccinated from eight to twelve weeks before the epidemic, however, essentially no protection was shown.[16]

The vaccination studies by Francis[3] and Hirst[4] and their associates during the influenza B epidemic of 1945 again yielded favorable results. The vaccinations were carried out within a month preceding the outbreak. Francis and

co-workers observed an incidence of 1.15 per cent among the vaccinated and 9.91 per cent among the unvaccinated individuals, while Hirst noted an incidence of 0.5 and 12.5 per cent respectively in the test and control groups. The virus strains isolated during the 1943 influenza A and 1945 influenza B epidemics were found to be closely related antigenically to those in the vaccines, and the significantly lower incidence among the vaccinated individuals was considered to be due to the prophylactic effect of vaccination.

During the 1947 influenza A epidemic, as shown in this and other reported studies, no such dramatic protection following vaccination was observed.[9-15] At the University of Michigan, Francis, Salk, and Quilligan[10] reported an incidence of influenza in 10,328 vaccinated individuals of 7.2 per cent compared with 8.1 per cent in 7,615 controls. Likewise in a study at Bucknell University by Fowle and associates, the incidence was 7.05 per cent in vaccinated persons (1,250) and 7.3 per cent in the unvaccinated (794).[11] The findings in these studies also support the epidemiological observations made by the Army during the 1947 epidemic among its personnel.[9] Van Ravenswaay[13] reported an incidence of 20.2 per cent among 237 vaccinated cadets compared with 27.8 per cent among 284 nonimmunized boys. Sigel and associates[14] studied the 1947 epidemic outbreak among 521 students in a boys' school, 88 per cent of whom were vaccinated. Of the vaccinated group, 54 per cent were considered to have become ill, whereas 49 per cent of the smaller control group contracted influenza. The incidence of moderately ill patients in the vaccinated and unvaccinated groups was 36 and 30 per cent respectively. Weller, Cheever, and Enders[15] also found no prophylactic effect following the intradermal inoculation of influenza A and B vaccine during the 1947 epidemic, although the antibody response to vaccine by this route appeared to be adequate. The epidemiological data in this study were obtained by questionnaire. Of the 316 individuals who received the vaccine, 34 per cent reported symptoms of acute upper respiratory tract disease, while 28 per cent of 329 unvaccinated personnel had a similar illness during the epidemic period.

The only favorable study reported to date on the value of vaccination against epidemic influenza during 1947 is that of Trimble.[12] This was carried out among the student population at the University of Missouri. The interpretation of the results of this study is open to question because the incidence of influenza among the vaccinated and unvaccinated groups was obtained by polling "at random various students encountered throughout the University campus and surroundings." A detailed analysis of the 880 cases admitted to the hospital during the epidemic period would have yielded more definitive results.

In our study the questionnaire method yielded a crude incidence of acute respiratory disease among the vaccinated and nonvaccinated groups of 46.5 and 41.5 per cent, respectively, whereas the incidence during the epidemic period was the same and was only 9.5 per cent in the test and control groups as shown by the clinic data. It was still lower as far as our serologic studies were concerned. Only 38.6 per cent of 148 pairs of sera collected from students showing symptoms of influenza during the epidemic period showed significant rises

(fourfold) in titer to the PR-8 diagnostic antigen. The studies of Sigel and associates[14] employing the complement fixation test and soluble PR-8 antigens[17] showed a much greater number with significant rises.

It is evident from the etiological studies of the influenza epidemic of 1947 that the failure of the combined A and B vaccines to protect was not due to the time interval between vaccination and the outbreak. Vaccination was equally ineffective as reported by others when done from two weeks to two and three months before influenza occurred. Although in our study the interval was from four to five months, antibody determinations on acute sera in vaccinated individuals showed essentially the same high titers as did the sera taken two to three weeks after vaccination. These observations support those of Francis, Salk, and Quilligan.[10] In all probability the failure of the vaccine in this epidemic was due, as Francis and associates have pointed out, "to the lack of sufficient antigenic crossing between strains of virus in the vaccine and the prevalent strains responsible for the epidemic." This conclusion was based on evidence obtained from comparative serologic studies with strains isolated during the 1947 epidemic and those incorporated in the vaccines. Our studies on the antigenic relationship between the recently isolated strains and those making up the vaccines support the observations of Francis and associates,[10] as well as those of Smadel,[9] Hirst,[18] and Sigel and co-workers.[19] The failure to demonstrate the usefulness of the vaccine in this epidemic should not be taken as a reflection on the vaccine itself or on previous studies during which positive results were obtained. The vaccination programs of Francis, Salk, Hirst, and other members of the Influenza Commission during the 1943 and 1945 epidemics establish without doubt the value of vaccination as a control procedure against the disease caused by virus strains closely related to those in the vaccine.[1-5]

The low antibody levels against the 1947 strains in the prevaccination and acute sera indicate that the population has had little previous experience with them. Whether the 1947 strains, which were seeded throughout the population during the spring months, will be responsible for subsequent outbreaks cannot be determined. Antibody response to vaccination[20] with combined influenza virus A and B vaccines containing, in addition to the PR-8, Weiss, and Lee strains, the FM-1 1947 strain was disappointing. While the PR-8, Weiss, and Lee strains stimulate significant antibody response in individuals with low initial titers (16 to 64) as shown by the chicken red cell agglutination inhibition technique, the FM-1 antigen produced little or no specific antibody rise. These studies point up the fact, as Van Ravenswaay concludes,[13] that "further study is necessary to define the application and limitations of influenza vaccine as a prophylactic agent." Sigel and co-workers offer suggestions for improving the vaccine.[14] Dingle,[21] in reviewing the problem of influenza, emphasizes the many gaps in our knowledge of this disease and its control.

CONCLUSIONS

No prophylactic effect was observed following vaccination with combined A and B influenza virus vaccines four to five months prior to an outbreak of

influenza among the students at the University of Chicago in March, 1947. The time between vaccination and the onset of the epidemic was probably not important in accounting for the lack of effect of the vaccines. Of most importance was the absence of a close antigenic relationship between the viruses in the vaccine and the strains causing the epidemic; this has been demonstrated by virus isolation and serologic studies.

Failure to demonstrate the usefulness of the vaccine during the 1947 epidemic should not be taken as a reflection on the vaccine or on previous studies during which positive results were obtained. Studies made during the 1943 and 1945 epidemics established without doubt the value of vaccination as a control procedure against the disease caused by a virus strain closely related to the strains in the vaccines. However, a comparison of data concerning the value of vaccination during this and the 1943 and 1945 epidemics shows clearly that a vaccine which affords protection during one epidemic does not insure protection against subsequent outbreaks.

The efficiency of a vaccine will depend (1) on its ability to initiate an adequate antibody response and (2) on a close antigenic relationship between the virus components in the vaccine and those initiating the epidemic. Further development of an influenza vaccine of broad antigenic coverage and study of the limitation of its usefulness as a prophylactic agent against epidemic influenza are needed.

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TOPICAL APPLICATION OF SUBTILIN TO TUBERCULOUS LESIONS IN MAN*

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IN RECENT years attention has been focused upon the chemotherapeutic approach to the treatment of tuberculosis. Promin and its derivatives were found to be highly effective against *Mycobacterium tuberculosis* in vitro and in experimental animals, yet clinical experience failed to reveal demonstrable effects of these drugs on tuberculous infections in human beings. On the other hand, streptomycin, which was similarly effective in vitro and in animals, has proved efficacious in the treatment of certain phases of human tuberculosis. Final evaluation of an antituberculosis agent must be carried out after its use in the treatment of the disease in human beings.

In the past two years there have been several reports regarding basic investigations of a new antibiotic, subtilin, which has been effective against *Myco. tuberculosis* in vitro and to a limited extent in animal experiments. This is a preliminary report on the topical administration of subtilin to eight patients with tuberculous lesions. It is realized that no conclusions can be drawn from a report of this nature, but it was felt that this limited clinical experience should be made available to other investigators.

The antibiotic activity of *Bacillus subtilis* has been recognized for a number of years, and in 1944 subtilin, an antibiotic derived from a strain of *B. subtilis*, was announced by Jansen and Hirschmann.[1] Bacteriostatic and bactericidal action in vitro against *Myco. tuberculosis* was reported by Salle and Jann[2] and confirmed by Wong, Hambly, and Anderson.[3] Preliminary studies reported by Salle[4] indicated that experimental tuberculosis in guinea pigs might be favorably altered by administration of subtilin, but experimentation in vivo was retarded by certain physical properties of the drug.

The solubility of subtilin in physiologic saline solution and in human serum is approximately 0.05 per cent, although it is readily soluble in water.[5] As shown by Wilson, Lewis, and Humphreys,[6] subcutaneous or intramuscular injection of strong aqueous solutions into animals produces very low concentrations of the drug in the blood, and subtilin precipitates at the site of injection. The saline tissue fluids apparently precipitate the antibiotic, and subsequent absorption of the precipitate is exceedingly slow.

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*Part of a cooperative study with the Division of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco, under the direction of Dr. H. H. Anderson; the Department of Bacteriology, University of California, Los Angeles, under the direction of Dr. A. J. Salle; and members of the staff of the Western Regional Research Laboratory, Albany.

The drug has not been administered orally because tests in vitro have revealed that digestive enzymes destroy a considerable portion of subtilin's activity.[7]

Observations on chemical and physical properties of subtilin have been reported by Dimick, Alderton, Lewis, Lightbody and Fevold,[7] and more recently by Lewis.[8]

Since subtilin was relatively insoluble in physiologic saline solution, parenteral administration was considered impractical. Although it is fully realized that aerosol administration alone is by no means a satisfactory method of treatment of tuberculosis, it was felt that useful information might be gained by employing the drug topically, and the present study was undertaken.

Topical application was by the intraoral administration of nebulized solutions of subtilin to eight patients with laryngeal or endobronchial tuberculosis.

OBSERVATIONS IN EIGHT CASES

The patients ranged in age from 18 to 60 years and included members of both sexes. All were observed for at least six weeks prior to subtilin therapy. As shown in Table I, four of the patients had tuberculous laryngitis and one had endobronchial disease. Three other patients had moderately or far advanced pulmonary tuberculosis with cavitation, and one of these had a recent bronchogenic dissemination of disease. These latter three patients were considered to have a conspicuous bronchial factor in their pulmonary disease although lesions were not observed in the main stem bronchi at bronchoscopic examination, and subtilin therapy was directed toward presumed endobronchial lesions in the smaller bronchi.

Method of Administration.—At the beginning of the study subtilin* was supplied as a 0.25 per cent solution in 0.45 per cent sterile saline solution. Subsequently the strength of subtilin in the solution was increased and the saline concentration was necessarily decreased (due to the low solubility of subtilin in saline). The daily dose was divided into four to ten portions of 0.5 to 1.0 cubic centimeter. The portion was introduced into a nebulizer† attached to a standard oxygen tank and administered intraorally as described elsewhere.[9]

Later a 1.8 per cent aqueous solution of urea was used as the solvent in place of saline. This isotonic solution produced less tracheal irritation.

Both 4 and 6 per cent solutions of subtilin were administered to one patient.

Dosage.—Early in the investigation patients were given 10 mg. of subtilin daily, and the dose was increased gradually in the course of two or three months to at least 90 mg. daily. For a considerable period two patients received 90 mg. daily, four patients, 120 mg. daily, and one patient, 160 mg. daily. One patient received 600 mg. daily for six weeks.

Clinical and Laboratory Studies.—In an effort to determine the action and possible toxicity of subtilin, the following laboratory procedures were performed

*The subtilin used in these studies was supplied by the Western Regional Research Laboratory, Albany, Calif.

†Kindly supplied by Vaponefrin Company, Chicago, Ill.

in addition to complete physical, bronchoscopic, x-ray, and fluoroscopic examinations: determination of vital capacity, measurement of daily sputum output, urinalysis, complete blood cell count, determination of sedimentation rate and of the concentration of nonprotein nitrogen and sugar in the blood and protein in the serum, albumin-globulin ratio, icterus index, and cephalin flocculation, bromsulfalein liver function test, and phenolsulfonphthalein renal function test.

Acid-fast bacilli were cultured from the sputum of all patients, with one exception, before therapy. Organisms were subsequently recovered from the sputum of this patient, and two years prior to subtilin therapy laryngeal biopsy revealed tuberculosis.

Sensitivity of the tubercle bacilli to the antimicrobial action of subtilin was determined in six cases prior to treatment.

During therapy the following procedures were carried out at weekly intervals: measurement of sputum output, culture and smear of three-day sputum concentrate, determination of vital capacity and sedimentation rate, complete blood cell count, and urinalysis. Chemical analyses of blood were repeated at monthly intervals.

The progress of the disease was followed during therapy by means of frequent physical, fluoroscopic, and x-ray examinations.

TABLE I. SUBTILIN INHALATION THERAPY: DATA OBTAINED FROM EIGHT PATIENTS

PATIENT	CONDITION	SUBTILIN (TOTAL GM.)	DURATION OF THERAPY (MO.)	IMPROVEMENT
1	Tuberculous laryngitis	3.8	3	Marked
2	Tuberculous laryngitis	51.4	6	Slight
3	Tuberculous laryngitis	17.5	7	Questionable
4	Tuberculous laryngitis	12.0	2½	None
5	Endobronchial tuberculosis	22.8	10	Marked at first, reactivation later
6	Presumed endobronchial tuberculosis	6.3	4	None
7	Presumed endobronchial tuberculosis	15.7	6½	None
8	Presumed endobronchial tuberculosis	7.7	4	None

Clinical Observations.—

Laryngitis: In the four patients with tuberculous laryngitis, improvement was marked in one, slight in one, and questionable in one. In the fourth, the disease progressed and the patient died.

The patient who showed marked improvement (Patient 1, Table I) had moderately advanced pulmonary tuberculosis with cavitation. Prior to subtilin therapy this patient had undergone therapeutic pneumothorax and phrenicotripsy. The laryngeal lesions had been treated previously with penicillin inhalation and local application of sulfadiazine crystals in an effort to control secondary infection, and the disease process revealed slight improvement prior to application of subtilin. A large cavity had not responded to pneumothorax. The laryngitis improved rapidly after institution of subtilin therapy, and after four months only scarring of the vocal cords remained. No change in the larynx occurred in six months thereafter, although reactivation of pulmonary disease

occurred. Since marked improvement in the laryngeal lesions occurred within one week of the beginning of subtilin therapy and while the dose was only 10 mg. daily, the relationship of the antimicrobial therapy to the disease regression is difficult to evaluate.

The patient whose condition showed slight improvement (Patient 2) had active pulmonary disease. After two and a half months of subtilin therapy there was moderate subjective and slight objective improvement. Despite continuation of subtilin therapy for a total of six months, no further improvement occurred.

The patient showing questionable improvement (Patient 3) had far advanced pulmonary tuberculosis confined to the right lung. Subtilin inhalations were originally instituted to observe the possible effect on the pulmonary lesions. Despite the development and subsidence of a right pleural effusion during subtilin therapy, the parenchymal disease showed slight gradual healing in x-ray studies. Hoarseness also developed while this patient was being treated, and laryngoscopic examination revealed lesions consistent with tuberculous laryngitis. Symptoms remained mild while use of the drug was continued, but after discontinuance the laryngeal disease showed progression, both subjectively and objectively. Whether this demonstrates any effect of subtilin on the laryngeal lesions is very doubtful.

Patient 4 had advanced pulmonary tuberculosis complicated by very severe and deeply infiltrated tuberculous laryngitis and, as later shown at autopsy, obstructive involvement of the esophagus. The laryngitis progressed during subtilin therapy. The possibility of influencing a process of this nature by topical application of a drug to the larynx is unlikely.

Endobronchial Disease: Therapy in one subject (Patient 5) could be carefully evaluated because of extensive lesions visible at bronchoscopy. Before treatment the left main stem bronchus of this patient was 80 per cent occluded by tuberculous granulations and debris. After three and a half months of subtilin therapy, during which the dose was gradually increased to 30 mg. daily, bronchoscopy revealed definite evidence of healing and only 20 per cent occlusion. After eight and a half months of therapy, during which the dose was increased to 120 mg. daily, bronchoscopy revealed no occlusion or other evidence of endobronchial disease. During subtilin therapy there was no appreciable change in the far advanced bilateral pulmonary tuberculosis, no increase in vital capacity (900 c.c.), and no conversion of the positive results of examination of the sputum. After ten months of subtilin therapy, another bronchoscopic examination revealed inflammation and granulations involving pharynx, larynx, trachea, and bronchi. This was interpreted as being tuberculous and recent in origin. Sputum cultures at this time revealed *Myco. tuberculosis* organisms which were twice as resistant to subtilin as organisms recovered in this case before treatment. It seems possible that subtilin favorably influenced the endobronchial disease originally, but it had no demonstrable effect on the pulmonary parenchymal disease. Although the development of marked bacterial resistance to subtilin was suspected, this was not proved by cultural studies, and the cause of the reactivation of endobronchial disease is unknown.

The condition in the three patients in whom endobronchial tuberculosis was presumed to be present in the small bronchi (Patients 6, 7, and 8) showed no significant change during subtilin therapy. In the absence of visible endobronchial lesions, evaluation of therapy was based on clinical condition and roentgenographic change in parenchymal disease. While there was no radiologic evidence of improvement in the extensive pulmonary disease, progression of parenchymal lesions was not demonstrated. In the patient in whom bronchogenic dissemination had occurred (Patient 8), the disease had been progressive at the beginning of administration of subtilin.

Laboratory Studies.—Strains of *Myco. tuberculosis* isolated from six of the eight patients prior to therapy showed no growth in subtilin dilutions of 1:400,000 (Patients 5, 6, and 8), 1:200,000 (Patients 3 and 7), and 1:100,000 (Patient 1).* After three and a half months of therapy the organisms isolated from three of these six patients (Patients 6, 7, and 8) showed no growth in solutions of subtilin four times the original effective concentrations. This decrease in bacterial sensitivity is probably beyond the experimental error of the procedure and is considered suggestive evidence of the development of bacterial resistance to subtilin in vivo. In one subject (Patient 5) only a twofold decrease in sensitivity was demonstrable after eight and a half months of therapy. This change may be within the experimental error of the test.

Determination of the concentration of subtilin in the blood was carried out in a patient who had received the drug by inhalation for three months, in the last two weeks of which the dose had been 400 mg. daily. No subtilin activity could be demonstrated in the blood.[10]

Toxicity.—No evidence of toxicity in the kidneys, liver, or bone marrow was encountered.

Administration of subtilin by inhalation was associated with irritation of the respiratory tree in almost all patients. Mild dyspnea, increase in cough and sputum, and mild pharyngitis occurred commonly during the first two or three weeks, after which the symptoms subsided or disappeared. The dyspnea and cough were frequently relieved by including from 2 to 4 drops of 0.25 per cent aqueous solution of Neosynephrin hydrochloride in the nebulized solution. Frank wheezing and sibilant râles were not associated with this reaction. In one case recurrent hemoptysis was encountered and in several others isolated episodes occurred.

Recurrent headaches developed in one patient receiving subtilin, and there were several isolated instances of headache among the patients receiving the drug. No neurologic abnormalities were found, and when headache was encountered it responded to the usual medications. Implication of subtilin as a causative agent does not seem warranted.

Asthma developed in one subject (Patient 1) after three months of therapy, and it was necessary to discontinue administration of the drug for this reason. No other evidence of hypersensitivity was observed.

It should be emphasized that the lack of serious toxicity encountered in the clinical use of this drug may be inherent in the mode of administration, which results in negligible systemic absorption of subtilin.

*We are indebted to Miss Joyce Amluxen for these studies.

COMMENT

It is fully realized that a drug should be administered systemically in order to exert a significant bacteriostatic or bactericidal effect in a disease such as tuberculosis in man. It is also fully appreciated that a large number of patients should be treated before any definite statement can be made regarding the clinical value of an antituberculosis agent. This preliminary investigation of the topical application of subtilin in a small number of cases does not warrant drawing any conclusions. However, suggestive evidence of therapeutic activity and of minimal toxicity affords stimulus for continued research.

Basic investigation is being directed toward the development of a modification of subtilin that can be administered parenterally. Intravenous infusion of very dilute solutions is possible in animals,[11] but utilization of this technique in man for a prolonged period would be impractical. The development of derivatives of subtilin with increased solubility in physiologic fluids and with retention of potency is being investigated by Lewis and associates. Preliminary results with some of these derivatives are encouraging. Work is also being carried on by Maclay and others regarding the possibility of combining subtilin with pectin to alter its characteristics of absorption.

Further investigation of the therapeutic possibilities of subtilin must await basic research directed toward development of derivatives more soluble in physiologic fluids. Subtilin in its present form has no place in the treatment of tuberculosis other than for purely investigative purposes.

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ADMINISTRATION OF PENICILLIN AND STREPTOMYCIN BY MEANS OF THE HYPOSPRAY APPARATUS (JET INJECTION); ABSORPTION, TOXICITY, AND STABILITY

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RECENTLY a device for the parenteral administration of solutions or suspensions of drugs known as the Microjet or Hypospray became available for study. Preliminary reports on its use clinically have been published by Hingson and Hughes[1] and by Hingson and associates.[2] The principle of the instrument is based upon the fact that an extremely fine high-pressure jet is capable of piercing the human skin with, at most, only slight pain. The instrument injects the drug in solution or suspension by means of high pressure* obtained through the release, by means of a button, of a previously wound calibrated high-tension spring which is attached to a plunger. The material to be injected is placed in a metal ampule (Metapule). It has a capacity of 0.25 c.c. and is shaped like a blunt-nosed bullet with an orifice 0.003 inch in diameter in the rounded tip while the butt end is stoppered with a rubber plug. The tip of the Metapule is held against the skin at the site of the injection with the base locked securely in the apparatus. The plunger explodes against this rubber stopper which forces the material out of the Metapule and through the skin as a fine spray. The material is deposited subcutaneously and intramuscularly to depths varying from 0.2 to 2 cm., depending on the tension of the spring and the site of injection.[1] Since the orifice in the Metapule through which the medication is expelled is only 0.003 inch, the size of a human hair, pain is either nonexistent or very slight because there is little trauma to the tissues and a minimum of pain nerve fibers is stimulated. These factors are discussed in detail elsewhere.[1] Another advantage attributed to this instrument is that it eliminates the fear incident to injection by needle and syringe.

ABSORPTION

Since many infections amenable to penicillin and streptomycin therapy require injections at regular intervals throughout the day, the availability of this instrument for the administration of these antibiotics would considerably facilitate therapy. A study of the absorption of penicillin and streptomycin following intramuscular injection was undertaken. Doses of 50,000, 100,000, and 200,000 units of penicillin and of 0.05 and 0.1 Gm. of streptomycin were employed. The latter dose of both antibiotics was found to be the maximum amount of each drug that could be dissolved in 0.25 c.c. of solution (the capacity of a Metapule).

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*The theoretic pressure in the Metapule is about 274 kg. per square centimeter. This is proportional, however, to the much smaller pressure of 11 Gm. exerted on the fine jet or column of liquid in the orifice of the Metapule. The jet or column exerts a pressure varying from 2,300 to 3,500 pounds per square inch on the skin through which it makes a minute hole or channel.

The percentage of patients having assayable concentrations of penicillin in the blood following the injection of 50,000, 100,000, and 200,000 units of penicillin by means of the Hypospray is shown in Table I. Following the injection of 50,000 units, detectable concentrations in the blood were found in all of the patients at one hour and in 94 per cent at two hours. Measurable blood concentrations were found in only 63 per cent of the patients at the third hour and

TABLE I. PERCENTAGE OF PATIENTS WITH MEASURABLE CONCENTRATIONS IN THE BLOOD FOLLOWING THE INJECTION OF VARIOUS DOSES OF BUFFERED CRYSTALLINE SODIUM PENICILLIN G BY MEANS OF THE HYPOSPRAY APPARATUS

DOSE (UNITS)	PERCENTAGE OF PATIENTS					
	HOUR					
	1	2	3	4	6	8
50,000	100	94	63	31		
100,000		100		100	50	0
200,000				100	86	14

in 31 per cent at the fourth hour after injection. When 100,000 units were administered, assayable concentrations were detected in the blood of all patients at four hours, in 50 per cent at six hours, and in none of the patients at eight hours after administration. Of the patients who were given 200,000 units of penicillin by this method, measurable concentrations were demonstrated in the blood of all the patients at four hours and in 86 per cent six hours after injection. After eight hours only 14 per cent of the patients were found to have demonstrable penicillin concentrations in the blood.

In order to compare the height and duration of the concentration of penicillin in the blood following administration by the Hypospray with that by the

TABLE II. CONCENTRATIONS OF PENICILLIN IN THE BLOOD FOLLOWING THE INTRAMUSCULAR INJECTION OF 50,000 UNITS OF BUFFERED CRYSTALLINE SODIUM PENICILLIN G WITH THE HYPOSPRAY APPARATUS (0.25 C.C.) AND BY THE NEEDLE AND SYRINGE (1.5 C.C.)

PATIENT	HOUR		
	2	3	4
Hypospray apparatus			
1	.12	.06	.06
2	.12	.06	0
3	.25	.06	.06
4	.06	0	0
5	--	.06	--
6	--	.06	--
7	--	.12	--
8	.25	.06	.06
9	.06	0	0
10	.5	.06	.06
Per cent	100	80	57
Needle and syringe			
1	.12	.06	0
2	.06	0	0
3	.06	0	.06
4	.06	0	0
5	.12	0	0
6	.12	0	--
7	.12	.03	0
8	.06	0	0
Per cent	100	25	12

needle and syringe, 50,000 units were given to a group of patients by both methods, and blood for penicillin assay was taken two, three, and four hours thereafter. The volume of the penicillin solution was 1.5 c.c. when given by needle and syringe, and 0.25 c.c. when the Hypospray was employed. It can be seen from Table II that the concentrations obtained with the Hypospray were somewhat higher and more prolonged than those obtained with the use of the needle and syringe. Although the volumes of the solution injection were not the same, it is believed that that does not account for the differences. It is our opinion that the differences may be due to the fact that part of the penicillin injected with the Hypospray is deposited subcutaneously. It has been demonstrated that the concentrations of penicillin in the blood are prolonged following subcutaneous injection as compared with the other methods of parenteral injection.

TABLE III. AVERAGE CONCENTRATIONS OF STREPTOMYCIN IN THE BLOOD FOLLOWING THE INJECTION OF VARIOUS DOSES OF STREPTOMYCIN BY MEANS OF THE HYPOSPRAY APPARATUS

DOSE (GM.)	AVERAGE CONCENTRATION IN THE BLOOD (μG./C.C.)		
	HOUR		
	4	8	12
0.05	1.25	.62	.31
0.1	2.5	1.25	.62

The mean concentrations of streptomycin in the blood at four, eight, and twelve hours following the injection of 0.05 and 0.1 Gm. of streptomycin are shown in Table III. At four, eight, and twelve hours after the administration of 0.05 Gm. of streptomycin, the mean concentrations were 1.25, 0.62, and 0.31 μg. per cubic centimeter of serum respectively. Following the injection of 0.1 Gm., the mean concentrations were 2.5, 1.25, and 0.625 μg. per cubic centimeter of serum at the same times.

TABLE IV. CONCENTRATIONS OF STREPTOMYCIN IN THE BLOOD FOLLOWING THE INJECTION OF 0.1 GM. STREPTOMYCIN EVERY TWELVE HOURS BY THE HYPOSPRAY APPARATUS AND WITH NEEDLE AND SYRINGE

PATIENT	CONCENTRATION IN THE BLOOD (μG./C.C.)							
	HOUR							
	12	24	36	48	60	72	84	96
Needle and syringe								
1	.6	.6	.6	.6	.6	.6	1.25	2.5
2	.6	.6	1.25	.6	.6	1.25	1.25	1.25
3	.3	.6	.6	.6	.6	.6	.6	.6
4	.6	.6	.6	.6	.6	.6	.6	.6
Hypospray apparatus								
1	.3	.6	1.25	.6	.6	.6	.6	.6
2	.6	1.25	.6	.6	1.25	1.25	.6	1.25
3	.3	.6	1.25	.6	.6	.3	.6	.3
4	2.5	2.5	2.5	5.0	1.25	1.25	5.0	2.5

Since significant concentrations were obtained twelve hours following the injection of 0.1 Gm. of streptomycin, it was decided to investigate the results of repeated injections of this dose at twelve-hour intervals and also to compare these results with the same dose given by needle and syringe. For this study,

blood for streptomycin assay was taken every twelve hours, immediately before the next injection (twelve hours following the preceding injection), over a period of four days. It can be seen from Table IV that there was no added effect from repeated injections and that the concentrations in the blood at the twelfth hour were at a plateau. Furthermore there was essentially no difference in the results obtained with the two methods of administration. Several patients were given 0.1 Gm. of streptomycin every four hours five times a day by means of the Hypospray. Assay of the blood at the fourth hour (immediately before the next injection) revealed constant concentrations at that hour, indicating no increment of streptomycin in the blood as a result of repeated injections.

The instrument employed in these studies had a spring which when released exerted a static pressure of 2,300 pounds per square inch on the skin. Although it has been shown that the depth and spread of the injected material varies with the site of injection and the race of the patient,[1] no differences in the blood concentrations of these antibiotics were noted in our series despite the fact that the sites of injection were varied (flexor and extensor aspects of arms, thighs, and buttocks) and both white and Negro patients were studied.

TOXICITY

Most patients complained of momentary stinging at the site of injection, particularly with the larger doses of penicillin and streptomycin. Of the sixty patients injected in this series, only four have shown an unfavorable reaction. One patient developed a small area of induration at the site of injection after twenty-four hours. This abated after twenty-four hours following treatment with warm, wet dressings. Two patients developed small hematomata at the sites of injection. A fourth patient developed a small, subcutaneous nodule at the site of injection several days after administration. This persisted for a period of two weeks. Similar reactions have been reported by others.[3] Although it is relatively simple to master the technique of administration with the Hypospray, small linear cuts, blister formation, and bleeding were obtained as the result of improper handling during the early use of this instrument. The area prepared for injection should be allowed to dry before administration is attempted in order to prevent the instrument from slipping on the skin.

One patient with tuberculosis was treated with five injections per day of 0.1 Gm. streptomycin every four hours. The posterior aspects of the arms and thighs were rotated as the site of injection. After about ten days of therapy, small nodules developed at the site of injection within twenty-four hours after each injection. It is believed that these reactions were the result of repeated subcutaneous depositions of part of the streptomycin during administration with the Hypospray apparatus. In support of this are the results of absorption studies, reported herein, which indicate that some of the drug was deposited subcutaneously.

There is also evidence to indicate that the high concentrations of both streptomycin and penicillin may have been a factor in the development of the few untoward local reactions observed with the use of the Hypospray in this series.

These studies were, for the most part, carried out on patients with tuberculosis. After the first few patients had received injections by this instrument, there was no problem in securing volunteers for the study in spite of the fact that several blood specimens were to be taken from each patient. This indeed testifies to the efficiency of this device, particularly when the attitude of these patients is considered.

STABILITY

Our data on the stability of several aqueous solutions of penicillin stored in Metapules and employed in these studies are shown in Table V. The instability of penicillin in aqueous solution is well established. These results indicate that the stability of aqueous solutions of penicillin stored in Metapules remains undependable. The apparent discrepancy in some of the results shown in Table V is undoubtedly explained by the facts that the hand method of filling the Metapules and the method of assay are subject to error.

TABLE V. STABILITY OF SOLUTIONS OF BUFFERED CRYSTALLINE SODIUM PENICILLIN G DISPENSED IN METAPULES (0.25 C.C. CAPACITY) EMPLOYED IN ADMINISTERING DRUGS BY MEANS OF THE HYPOSPRAY APPARATUS

LOT	DOSE (UNITS)	BASE ASSAY* (UNITS)	WEEKS AFTER BASE ASSAY†		
			1	5	10
			UNITS		
1	50,000	44,000	44,500	37,500	36,000
2	50,000	34,500		30,250	25,000
3	50,000	45,000		53,000	
4	100,000	94,000	84,000	115,000	50,000

*Two to three days after preparation; not refrigerated during interval.
†Refrigerated.

COMMENT

Hingson and associates[2] have demonstrated that gonorrhea can be treated as effectively with this apparatus as with needle and syringe. Dispensing penicillin in solution in Metapules at present for general commercial use with the present design of the apparatus is, at least, very impractical. With the available instrument the dose of the antibiotics is limited by the size of the Metapule. This method of administration is not feasible, particularly in the treatment of infections requiring large doses of streptomycin. It appears, however, that the problem of dispensing penicillin and streptomycin will be solved.[3] The instrument has been improved so that it will discharge the contents of 0.5 and 1 c.c. Metapules.

The possibility of drugs being deposited in blood vessels during the course of injection with the Hypospray was anticipated. In spite of deliberate efforts to deposit some of the drugs injected directly into blood vessels, this has been accomplished only once when injection was made directly over a vein.[3] With most drugs this would be relatively unimportant, but there are some drugs which would have serious consequences following injection directly into the circulation. Further investigation is necessary to resolve this problem completely.

SUMMARY

These studies are evidence that penicillin can be administered by means of the Hypospray apparatus. Furthermore the device has the advantage of decreasing the pain and fear of injection which attend available methods. There are several problems to be solved before the device can be used generally in clinical practice.

We wish to thank Dr. William A. Randall and Dr. Clifford V. Price and Miss Velma L. Chandler for their assistance in the conduct of these studies.

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THE USE OF YEAST PHASE ANTIGENS IN A COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS

I. Preliminary Results With Rabbit Sera

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THE coincidence of pulmonary calcifications and skin sensitivity to histoplasmin[1-5] has suggested either a higher rate of infection with *Histoplasma capsulatum* than previously recognized or infection with an immunologically cross-reacting antigen. A recent report[6] that *H. capsulatum* had been isolated from nonfatal cases implies that infection with this agent may not be uniformly fatal. The high prevalence of skin sensitive reactors to histoplasmin in the eastern central states warrants the search for additional tools for evaluating evidence of past or present infections with *H. capsulatum*. Immunologic procedures properly developed and controlled may offer additional information.

Skin test antigens (histoplasmin) are prepared from broth filtrates of the mycelial phase of *H. capsulatum*. Since it is the yeast phase of the organism that appears in affected tissues of man and animal, studies utilizing this phase of the infectious agent may yield additional and possibly more specific results. The ready growth and maintenance of *H. capsulatum* in the yeast phase has been reported previously by one of us (C.C.C.)[7] and by others.[8, 9] The utilization of this antigen in preliminary complement fixation studies in experimental animals is herein described. Both viable and nonviable antigens were used in immunization. Since no appreciable differences were noted other than a slightly greater immunogenic capacity of the viable forms, the experiments described below are those utilizing killed antigens.

MATERIALS AND METHODS

Antigens.—Three strains of *H. capsulatum* in the yeast phase which we call G-2, G-5, and G-6 were used.† Two strains of *Blastomyces dermatitidis* (A-1 and A-5), one strain of *Blastomyces brasiliensis* (B-2), and AMS E-11 strain of *Candida albicans* were also studied.‡ All of the cultures were grown in the yeast phase on glucose cystine agar slants at 37° C. for five to fifteen days. The growth was washed off with sterile buffered saline and the pooled washings were filtered through sterile gauze, placed in a sterile rubber-stoppered vial, and heated at 56° C. for four hours. This constituted the stock antigen. For each series of tests, calculated amounts of this stock suspension were withdrawn, washed

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†The G-2, G-5, and G-6 strains of *H. capsulatum* were reverted from the mycelial phase of strains 715, 952, and 650, respectively, from the collection of Dr. N. F. Conant at Duke University.

‡A-1 strain of *B. dermatitidis* and A-5 (Duke 930) and B-2 strain of *B. brasiliensis* (Duke 871B) are all isolates from human subjects received from Dr. Conant at Duke University. The E-11 strain of *C. albicans* was isolated from a patient at the Army Medical Center.

two times with buffered saline, and diluted for use. The optimal antigen dilution was then determined by titration as that amount which gave the highest titer with homologous antisera and was not hemolytic or anticomplementary. The stock antigen retains its titer for several weeks when stored in the refrigerator. The portions removed for the daily test were heated at 56° C. for thirty minutes to eliminate any possible anticomplementary activity.

Preparation of Sera.—Young, healthy, albino rabbits weighing 5 to 7 pounds were immunized with yeast phase antigens of *H. capsulatum* in the following manner: The organisms grown on cystine agar slants for five days at 37° C.[7] were washed off with 0.5 per cent formalized isotonic saline and stored overnight at 3 to 4° C. The suspensions were then washed three times with physiologic saline and resuspended in 0.1 per cent formalized saline to a turbidity equal to the No. 3 MacFarland nephelometer standard. Rabbits were injected intravenously for six successive days with the following amounts: 0.1, 0.2, 0.5, 1.0, 2.0, and 2.0 milliliters. Booster injections of 2 ml. were given one and two weeks after the last injection and the rabbits were bled six days after the last booster. The same procedure was used for the production of sera against the antigens of the other organisms used in this study.

Complement Fixation Test.—The complement fixation test of Kent and Rein,[10] developed at the Army Medical School, was employed in these studies.

Diluent: For all dilutions 0.85 per cent salt solution buffered to pH 7.3 with 0.005M phosphate was employed.

Sheep's Red Blood Cells: A suspension of 2 per cent washed packed cells was prepared from blood collected aseptically into an equal volume of modified Alsever's solution.[11]

Hemolysin: Hemolysin prepared by the immunization of rabbits was used in an optimal dilution beyond which further decrease in dilution failed to diminish the quantity of complement required for fifty per cent hemolysis.[12] An equal volume of this optimally diluted hemolysin was poured into the cell suspension and mixed by ten successive pourings and at least ten minutes at room temperature were allowed for sensitization.

Complement: Lyophilized commercial guinea pig serum, 7.0 ml., was rehydrated with 5.0 ml. of the accompanying preservative containing 2 per cent boric acid and 5 per cent sodium acetate. A stock 1:20 dilution was prepared by adding 19 parts of salt solution to 1 part of rehydrated serum. The complement was titrated by the method of Kent, Bukantz, and Rein[13] for the determination of the 50 per cent unit of complement, and 3 units were employed in the test.

TABLE I. COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS

	TEST		REAGENT CONTROLS		
	WITH ANTIGEN	SERUM CONTROL	ANTIGEN	COMPLEMENT	SENSITIZED CELLS
Serum dilutions (ml.)	0.2	0.2			
Complement (ml.) (3 units)	0.2	0.2	0.2	0.2	
Antigen (ml.)	0.2		0.2		
Salt solution (ml.)		0.2	0.2	0.4	0.6
Fixation overnight at 3-6° C.					
Sensitized cells (ml.)	0.4	0.4	0.4	0.4	0.4
Incubation for thirty minutes at 37° C.					

Degrees of reaction in tests are read according to the percentage of cells remaining unhemolyzed. The tube with the greatest dilution of serum showing 50 per cent or less of hemolysis represents the titer of the serum. It is recommended that color standards representing 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 per cent hemolysis be employed to assist in determining the aforementioned grades of reaction.

Test: The test was conducted with suitable controls as indicated in Table I. The reagents were added in the order given and the tubes thoroughly mixed. The tubes were then placed in the refrigerator at 3 to 6° C. overnight. Sheep's cells sensitized at least ten minutes previously were then added; the tubes were shaken and incubated in a 37° C. water bath for thirty minutes. The tubes were then centrifuged at 7,000 revolutions per minute

for five minutes and the tests were read against a diffuse source of light in terms of per cent hemolysis, using control standard tubes for comparison. The greatest serum dilutions showing 50 per cent or less of hemolysis represent the titer of the complement-fixing antibodies.

RESULTS

Experiment 1, Titration of Antigen.—Each lot of antigen was titrated to determine the optimal dilution. The antigenic unit was considered to be the greatest concentration contained in 0.2 ml. beyond which further increase failed to enhance appreciably the serum reaction. It was required that the optimal concentration be neither hemolytic nor anticomplementary. It was found that the optimal dilution usually lay between 1:20 and 1:50 of the stock solution, depending on the concentration of the latter. Table II demonstrates the determination of the optimal antigen dilution of the G-2 antigen. The tubes containing the 1:40 dilution of antigen showed no traces of anticomplementary activity and likewise reacted with the antiserum to give clear-cut fixation of complement. The next dilution of antigen (1:53.3) reacted even more favorably. Thus an intermediate dilution of 1:50 was selected arbitrarily as the optimal amount and was used in subsequent studies.

TABLE II. DETERMINATION OF THE OPTIMAL DILUTION OF ANTIGEN USING THE G-2 STRAIN OF H. CAPSULATUM AND SERUM PREPARED IN RABBITS AGAINST G-2

DILUTION OF ANTIGEN (0.2 ML.)	POSITIVE SERUM DILUTIONS IN PER CENT (0.2 ml.)					CONTROLS (%)		
	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
1:10	0	0	0	10	95	97	100	0
1:13.3	0	0	0	25	95	97		
1:20	0	0	0	25	95	97		
1:26.6	0	0	0	30	95	97		
1:40*	0	0	0	35	95	100		
1:53.3*	0	0	0	20	60	100		
1:80	0	0	0	30	90	100		
Serum control	100	100	100	100	100			

0, No hemolysis; 100, complete hemolysis.

*Optimal dilution lies between 1:40 and 1:53.3 since there is good fixation but no trace of anticomplementary activity. For tests, 1:50 was employed.

Experiment 2.—Three sets of rabbit antisera prepared against three strains of *H. capsulatum* (G-2, G-5, G-6) were titrated against homologous and heterologous strains of these organisms as well as against yeast phase antigens of two strains of *B. dermatitidis* and one of *B. brasiliensis*. Fixation of complement was obtained in relatively similar titers with all combinations of histoplasma yeast phase antigens and histoplasma antisera. For example, as noted in Table III, sera prepared against G-2 histoplasma antigen fixed complement in the presence of its specific antigen to a titer of 1:160. In the presence of the G-2 antigen, G-5 and G-6 antisera bound complement in dilutions of 1:80. Sera prepared against *C. albicans*, yeast phase *Sporotrichum schenckii**[14] and *Cryptococcus neoformans** gave negative complement fixation results in the presence of the histoplasma G-2 antigen, while *B. dermatitidis* serum fixed complement only in dilutions up to 1:10.

*Isolated from patient material at the Army Medical Center.

TABLE III. REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF THE G-2 STRAIN OF H. CAPSULATUM AS ANTIGEN AND THE SERA OF RABBITS INOCULATED WITH THE G-2, G-5, AND G-6 STRAINS OF H. CAPSULATUM, THE A-1 AND A-5 STRAINS OF B. DERMATITIDIS, B. BRASILIENSIS, S. SCHENCKII, C. NEOFORMANS AND C. ALBICANS, AND NORMAL CONTROL RABBIT SERUM

	SERUM DILUTIONS (%)					CONTROLS (%)		
SERA	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
G-2	0	0	0	0	20	100	100	0
G-5	0	10	40	50	95	100		
G-6	0	0	10	40	95	100		
A-1	10	100	100	100	100	100		
A-5	10	100	100	100	100	100		
B. brasiliensis	95	100	100	100	100	100		
Sporotrichum	100	100	100	100	100	100		
Cryptococcus	90	100	100	100	100	100		
Candida	100	100	100	100	100	100		
Normal	100	100	100	100	100	100		

0, No hemolysis (complete fixation); 100, complete hemolysis (no fixation).
The titer was taken as being the highest dilution of serum showing 50 per cent or less of hemolysis.

When the G-5 antigen was used (Table IV), G-2, G-5, and G-6 antisera fixed complement in dilutions of 1:160, 1:80, and 1:80 respectively. Again no fixation was noted with sera prepared against the other fungi studied with the exception of *B. dermatitidis* serum which fixed complement in low titer. Similar results were obtained using G-6 antigen and homologous and heterologous sera.

TABLE IV. REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF THE G-5 STRAIN OF H. CAPSULATUM AS ANTIGEN AND THE SAME SERA USED IN TABLE III

	SERUM DILUTIONS (%)					CONTROLS (%)		
SERA	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
G-2	0	0	0	0	20	100	100	0
G-5	20	0	10	40	65	100		
G-6	0	0	0	15	70	100		
A-1	10	100	100	100	100	100		
A-5	10	95	100	100	100	100		
B. brasiliensis	95	100	100	100	100	100		
Sporotrichum	100	100	100	100	100	100		
Cryptococcus	90	100	100	100	100	100		
Candida	100	100	100	100	100	100		
Normal	100	100	100	100	100	100		

See footnotes to Table III.

Experiment 3.—Strains of blastomyces and histoplasma have been found to cross react in the skin testing of animals infected with these agents[15] as well as in complement fixation tests employing mycelial phase histoplasmin and blastomycin as antigens.[6, 16] In Experiment 2 it was noted that blastomyces antiserum in low dilution fixed complement in the presence of yeast phase antigens of *H. capsulatum*. In order more fully to evaluate this possible relationship, complement fixation tests were done employing yeast phase blastomyces antigens in the presence of blastomyces and histoplasma antisera.

In our hands the immunogenic capacity of *B. dermatitidis* in rabbits has been far inferior to that of *H. capsulatum*. Despite repeated booster injections the highest titer of complement-fixing antibodies was 1:40. When the A-5 antigen of *B. dermatitidis* was employed (Table V) in contrast to the 1:40 titers of the blastomyces antisera, the G-2, G-5, and G-6 histoplasma antisera fixed complement at dilutions of 1:80, 1:80 and 1:20 respectively. At first glance it would appear that the results obtained using blastomyces antigen show cross reactions too great to allow proper evaluation. However when the titers of the histoplasma antisera against the blastomyces antigen are evaluated in terms of their specific titer (compare Tables III and IV with Table V), it will be seen that the nonspecific titers are usually one-fourth to one-half of the specific titers, whereas the 1:40 titer obtained with blastomyces serum against its specific antigen actually represents maximum titer. Nevertheless the specificity of the

TABLE V. CROSS REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING THE A-5 STRAIN OF B. DERMATITIDIS AS ANTIGEN AND SERA OF RABBITS INOCULATED WITH THE A-1 AND A-5 BLASTOMYCES ANTIGENS AND THE G-2, G-5, AND G-6 HISTOPLASMA ANTIGENS

	SERUM DILUTIONS (%)					CONTROLS (%)		
SERA	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
A-1	0	10	25	100	100	100	100	0
A-5	0	0	10	100	100	100		
G-2	0	0	0	30	60	100		
G-5	0	0	0	45	95	100		
G-6	0	50	80	100	100	100		

See footnotes to Table III.

complement fixation test using histoplasma antigen in Experiment 2 was greater than that in which blastomyces antigen was used. This type of cross reaction suggests the presence of a common antigenic component shared by *H. capsulatum* and *B. dermatitidis* in which diluted histoplasma antiserum is capable of reacting with the histoplasma antigen carried by both organisms but in which blastomyces antiserum will not react as readily with histoplasma antigen. This may be due to differences in location of the common antigen in the respective antigenic patterns of *H. capsulatum* and *B. dermatitidis* and/or a greater immune response in experimentally infected animals to the former agent as shown by the greater immune response of rabbits to histoplasma antigen.

Experiment 4.—In the course of this study a report by Salvin[17] appeared describing the use of a yeast phase antigen of *H. capsulatum* in the Bengston complement fixation test.[18] No cross reactions between *H. capsulatum* and *B. dermatitidis* were noted, and details concerning titers or degrees of fixation were lacking. Since our technique differs in several fundamental respects from these methods, *H. capsulatum* and *B. dermatitidis* antigens were prepared according to Salvin's method and a comparison was made of the two types of complement fixation tests.

Table VI shows the results obtained when the complement fixation was done using Salvin's method in its entirety except that hemolysis was read in per cent. Using the G-2 histoplasma antigen and designating complete hemoly-

TABLE VI. BENGSTON'S COMPLEMENT FIXATION TEST UTILIZING THE G-6 HISTOPLASMA ANTIGEN PREPARED ACCORDING TO SALVIN'S METHOD

	SERUM DILUTIONS (%)					CONTROLS (%)		
SERA	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
G-2	45	90	95	80	95	100	100	0
G-5	90	90	90	95	95	100		
G-6	90	90	95	95	95	100		
A-1	95	95	95	100	100	100		
A-5	95	95	95	100	100	100		

See footnotes to Table III.

sis as the end point, titers of 1:160 were obtained with all three strains of histoplasma antiserum, while the blastomyces antisera against the A-1 and A-5 strains both yielded titers of 1:40. The degrees of hemolysis, however, between two-fold dilutions did not vary appreciably. This was particularly true with the G-5, G-6, A-1, and A-5 antisera where the hemolytic range was limited between 90 and 100 per cent. Two factors probably are involved in this type of reaction: (1) excess of complement; two full units used in this test represent approximately 5.1 50 per cent units as calculated by means of the Von Krogh alternation formula[19] taking a mean value of 0.175 for the constant 1/n; this represents almost double the amount of complement used in our test (3 50 per cent units); (2) incomplete fixation at the short one-hour incubation period.

TABLE VII. COMPARATIVE EFFECTS OF THE INCUBATION PERIOD ON COMPLEMENT FIXATION; SET A INCUBATED AT 37° C. FOR ONE HOUR; SET B INCUBATED OVERNIGHT AT 3 TO 6° C.; G-6 ANTIGEN USED IN BOTH SETS

SET A—FIXATION AT 37° C. FOR ONE HOUR								
	SERUM DILUTIONS (%)					CONTROLS (%)		
SERA	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
G-2	0	10	30	60	100	100	100	0
G-5	10	30	80	90	100	100		
G-6	30	50	50	100	100	100		
A-1	95	95	95	100	100	100		
A-5	95	100	100	100	100	100		
SET B—FIXATION OVERNIGHT AT 3 TO 6° C.								
G-2	0	0	0	15	50	100	100	0
G-5	20	0	10	40	65	100		
G-6	0	0	5	60	90	100		
A-1	100	100	100	100	100	100		
A-5	100	100	100	100	100	100		

See footnotes to Table III.

To evaluate further the second factor, that is the incubation period, two parallel series, A and B, were set up using the G-6 antigen and G-2, G-5, G-6, A-1, and A-5 antisera in the manner used routinely in our test. As noted in Table VII, fixation of set A was carried out at 37° C. for one hour while set B was incubated overnight at 3 to 6° C. Definitely stronger and more clear-cut fixation was obtained in the set incubated in the refrigerator overnight.

DISCUSSION

The development and use of a stable specific yeast phase antigen of *H. capsulatum* in complement fixation studies using immune rabbit sera is herein

described. Rabbits immunized with yeast phase *H. capsulatum* antigens developed complement-fixing antibodies in high titer reacting with homologous and heterologous strains of histoplasma. No fixation occurred when antisera against *C. albicans*, *C. neoformans*, *S. schenckii*, and *B. brasiliensis* were used. However, antisera against *B. dermatitidis* having a specific titer of 1:40 fixed complement in the presence of histoplasma antigens in low dilutions of serum. When blastomyces antigens were used, however, the cross reactions, observed to exist only in the smaller dilutions between histoplasma and blastomyces antisera, were more pronounced. In fact, because of the weaker immunizing capacity of the blastomyces antigen as compared with the histoplasma antigen, comparable or even higher titers were observed with the histoplasma antisera. However, these titers of histoplasma antisera against blastomyces antigen were usually one-fourth to one-half of the titer obtained when histoplasma antigen was used. For example, histoplasma sera with a titer of 1:160 fixed complement in the presence of the blastomyces antigen up to dilutions of 1:40. Although numerically this was equivalent to the 1:40 titer of the specific blastomyces antisera, the latter represented the maximum specific titer obtainable under the conditions of our test. This suggests the presence of a common cross reacting antigen in both *B. dermatitidis* and *H. capsulatum* which confirms the observations made on the basis of skin tests in infected animals by Howell[15] and in complement fixation tests by Tenenberg and Howell[16] and Bunnell and Furcolow[6] in which filtrates of the mycelial phase of histoplasma and blastomyces were used as antigens. The results of the complement fixation tests can be more accurately evaluated by the joint use of properly prepared *H. capsulatum* and *B. dermatitidis* antigens and serial dilutions of the test sera.

The yeast phase antigen of *H. capsulatum* adapts itself readily to use in the complement fixation test of Kent and Rein[10] in which excess of complement is avoided, adequate fixation is allowed, and the per cent hemolysis is utilized to obtain clear-cut, specific results. We were unable to confirm Salvin's findings that no cross exists between *H. capsulatum* and *B. dermatitidis* in the yeast phase either by use of the Bengston complement fixation test[17] or by that employed in this laboratory. Our observations are more in accord with others[6, 15, 16] that immunologic cross reactions between these two agents do exist. This would imply that in complement fixation tests for either human histoplasmosis or blastomycosis both antigens should be employed.

SUMMARY

A method employing yeast phase antigens of *H. capsulatum* in complement fixation tests with immune rabbit sera is described.

The Kent and Rein complement fixation test utilizing per cent hemolysis was clear cut and definite.

An immunologic relationship between *H. capsulatum* and *B. dermatitidis* was demonstrated.

The potential value of employing yeast phase antigens in complement fixation tests for the diagnosis of past or present infections with histoplasmosis

as a possible adjunct to the present methods now available is open to investigation.

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THE CONCENTRATION OF THE LABILE FACTOR OF THE PROTHROMBIN COMPLEX IN HUMAN, DOG, AND RABBIT BLOOD; ITS SIGNIFICANCE IN THE DETERMINATION OF PROTHROMBIN ACTIVITY

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THE prothrombin time, which may be defined as the coagulation time of plasma to which an excess of thromboplastin has been added, is generally accepted as a reliable quantitative measure of prothrombin activity. Until recently prothrombin was considered a unitary substance and any diminution of prothrombin activity was interpreted as a specific decrease of this clotting agent. In 1943 one of us (A. J. Q.) found that the loss of prothrombin activity in stored plasma was not due to a diminution of the classic prothrombin, which is adsorbable by $Al(OH)_3$ and which diminishes in dicumarol poisoning, but to that of a new factor not previously recognized.[1] This substance was named component A of prothrombin, but when it was later found that the adsorbable fraction of prothrombin contained two components, the terms components A and B were applied to these and the substance which disappears in stored plasma was redesignated the labile factor.[2]

Soon after these studies were published, Owren[3] independently discovered a similar clotting agent which he named factor V. He found that this factor was lacking in a patient with a serious hemorrhagic diathesis. In retrospect it seems probable that the patient of Rhoads and Fitz-Hugh,[4] diagnosed as having idiopathic hypoprothrombinemia, likewise suffered from a lack of the labile factor. Since diminution of this agent causes marked impairment of coagulation and a concomitant bleeding condition, it seems obvious that the labile factor is indispensable for coagulation or, more specifically, for prothrombin activity.

It becomes of great practical importance to know how this new factor affects the prothrombin determination. In this study therefore an attempt has been made to obtain information on that question. So far no means have been found to alter the concentration of this agent in vivo, and clinical study too is limited since Owren's case is the only one known at present in which the factor appeared diminished. Stored plasma from which the labile factor has been markedly reduced is the only available means for the assay of the substance in various types of plasma and for studying quantitatively its effect on the prothrombin time. Since human blood contains a relatively low concentration of the labile factor, storage will bring about a prompt and striking depletion.

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and therefore aged oxalated human plasma was found satisfactory for the present studies. Storage of plasma in open test tubes at ordinary refrigerator temperatures (4° C.) for seven days usually resulted in an increase of the prothrombin time from a normal of 12 seconds to 40 or more. No attempt was made to isolate the labile factor since it was not considered essential for the objective of the present study. By treating the plasma to be assayed with tricalcium phosphate, both components A and B are removed, thus leaving, as the only known plasma constituents playing a role in the process of clotting, fibrinogen and the labile factor.

MATERIAL AND METHODS

The Concentration of the Labile Factor in Human, Dog, and Rabbit Plasma.—To assay blood for its content of labile factor, it was collected by venipuncture and nine volumes immediately were mixed with one volume of 0.1M sodium oxalate. The plasma was treated with tricalcium phosphate* as follows: 1 c.c. of a 0.008M suspension of tricalcium phosphate was transferred to a small test tube and centrifuged. The supernatant water was poured off and the tube drained. One cubic centimeter of plasma was then mixed with the packed tricalcium phosphate. After ten minutes of incubation at room temperature the adsorbent was separated by centrifugation. The plasma obtained no longer clotted when mixed with thromboplastin and calcium chloride. By this procedure components A and B are removed, but the concentration of the labile factor is only minimally affected, as determined by preliminary studies.

On adding this treated plasma to stored human plasma, the prothrombin time is strikingly shortened. By determining the amount of plasma that has to

TABLE I. THE EFFECT ON THE PROTHROMBIN TIME RESULTING FROM THE ADDITION OF VARYING AMOUNTS OF THE LABILE FACTOR (PRESENT IN FRESH AND STORED PLASMA) TO STORED HUMAN PLASMA

Volume of stored human plasma	100	99.8	99	98	90	80	70	60	50	40	30	20	10
Volume of plasma assayed for labile factor:*		0.2	1	2	10	20	30	40	50	60	70	80	90
	PROTHROMBIN TIME (SEC.)												
Rabbit, fresh	71	20	15	10½	8½	8	7	7	7	7	8	9	11½
Dog, fresh	71		20	16	11	8½	8½	8½	8½	8½	8½	9½	14
Human, fresh	71				21	15½	14	13	12	12	12	13	20
Rabbit, stored (17 days)†	71			19	13	11	10	9½	9½	9½	9½	11	15
Dog, stored (17 days)†	71				22	17½	17	16	15	15	15	17	24
Human, stored (6 days)†	71				27	27½	29	30	33	35	37	44	67

*The plasma was treated with $Ca_3(PO_4)_2$ to remove components A and B.

†The prothrombin times of the stored plasma were: rabbit plasma, 10 sec.; dog plasma, 22 sec.; and human plasma, 26 seconds.

*To prepare tricalcium phosphate suspension: 158 Gm. of trisodium phosphate are dissolved in 1,000 c.c. of distilled water. Separately, 66.6 Gm. of anhydrous calcium chloride are dissolved in 1,000 c.c. of distilled water. The calcium chloride solution is poured into the trisodium phosphate with thorough stirring. The pH is adjusted to 7. The precipitated tricalcium phosphate is washed repeatedly by decantation until the sodium chloride is removed. The volume is brought to 1 L., thus making a 0.2M suspension. From this stock a 0.008M solution is prepared by mixing 4 c.c. with 96 c.c. of distilled water.

be added to a fixed amount of stored plasma in order to reduce the prothrombin time to an arbitrarily selected value, the relative concentration of the labile factor can be calculated. The value taken as standard was 20 seconds, the prothrombin time which is obtained when fresh oxalated human plasma treated with tricalcium phosphate is added to stored oxalated human plasma in the proportion of 1 to 10. It can be seen from Table I that only one part of rabbit plasma needs to be mixed with 500 parts of stored plasma to reduce the prothrombin time to 20 seconds, while dog plasma will bring about the same reduction in a dilution of 1 to 100. These findings indicate that fresh rabbit blood contains fifty times and dog blood ten times as much labile factor as human blood.

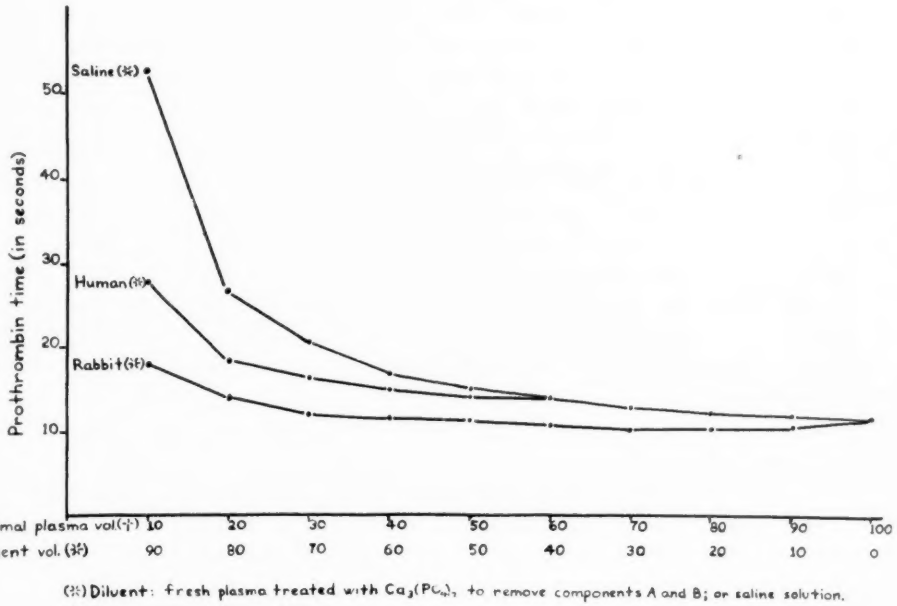
Saline (%)
Human (%)
Rabbit (%)
Prothrombin time (in seconds)
50
40
30
20
10
Normal plasma vol. (†) 10 20 30 40 50 60 70 80 90 100
Diluent vol. (%) 90 80 70 60 50 40 30 20 10 0
(%) Diluent: fresh plasma treated with $Ca_3(PO_4)_2$ to remove components A and B; or saline solution.
(†) Fresh oxalated human plasma.

Fig. 1.—The influence of varying concentrations of the labile factor on the prothrombin time of fresh normal human plasma.

When rabbit blood was stored for seventeen days, the labile factor decreased to such an extent that a dilution of 1 to 50 was required to obtain a prothrombin time of 19 seconds. Apparently the labile factor decreased approximately to one-tenth of its original concentration in seventeen days of storage. A similar decrease occurred in stored dog plasma. Due to the initial high concentration in rabbit and dog plasma, the effect of the loss of labile factor in storage as indicated by an increase in prothrombin time is not so striking as in human plasma.

It will be observed that as the volume of fresh plasma treated with tricalcium phosphate added to stored plasma is increased, the prothrombin time decreases to a minimal value that remains constant over a rather wide range (Table I). Munro and Munro[5] reported similar findings. The higher the concentration of the labile factor, the lower the minimal constant value. Thus

rabbit plasma reduces the prothrombin time of stored oxalated human plasma to 7 seconds, whereas human plasma lowers it only to 12 seconds.

The Effect of Different Concentrations of the Labile Factor on the Prothrombin Time of Fresh Human Plasma.—In 1945 one of us (A. J. Q.) observed that the prothrombin time of fresh human plasma diluted with progressively increasing amounts of rabbit plasma treated with aluminum hydroxide did not give a typical hyperbolic curve such as is obtained by dilution with saline, but one in which a minimal value was quickly attained and which remained unchanged over a wide range of dilutions.[6] Prothrombin curves obtained by using saline or fresh human or rabbit plasma treated with tricalcium phosphate as the diluent of fresh oxalated human plasma are shown in Fig. 1. It seems definite that the high concentration of the labile factor was responsible for the shape of the curve obtained with rabbit plasma, because when fresh human plasma treated with tricalcium phosphate was employed as diluent, thereby keeping the concentration of the labile factor low and constant, the curve approximated that obtained with saline. It is not possible to explain the action of the labile factor or the shape of the curves, but it is obvious that the factor is a key substance in the formation of thrombin.

DISCUSSION

One of the most striking observations recorded in this study is the low concentration of the labile factor in human blood. It is certain that this accounts in part for the relatively long prothrombin time of human plasma as compared with that of many of the common laboratory animals such as the rabbit and the dog. But there must be other factors, as shown by the result of the simple experiment of mixing oxalated human plasma with an equal volume of fresh rabbit plasma treated with tricalcium phosphate to remove component A and B but not the labile factor nor fibrinogen. In this mixture the concentration of the labile factor was increased about twenty-five fold, yet the prothrombin time was decreased only from 12 to 10 seconds. If rabbit plasma is diluted with an equal volume of fresh human tricalcium phosphate treated plasma, which produces a mixture having about the same concentration of labile factor, a prothrombin time of 7 seconds is obtained. Since the prothrombin times are not a linear function of prothrombin activity but vary according to the well-known hyperbolic curve, and since these values of prothrombin time are in the steep part of the curve, the difference between 10 and 7 seconds is much greater than would be shown by the simple arithmetic ratio.

Studies on the concentration of the labile factor in healthy subjects show that it is remarkably constant. No means so far has been found to alter specifically and exclusively its concentration. It still remains to be determined whether its level is altered in certain pathologic conditions. In the well-recognized types of hypoprothrombinemia such as vitamin K deficiency, dicumarol poisoning, and hereditary deficiency of either component A or B, no significant change in the concentration of the labile factor occurs. In recent years cases of hyperprothrombinemia have been reported. Many of these are based on prothrombin time determinations of highly diluted plasma, often with complete

disregard of the fibrinogen concentration, which, as Deutsch and Gerarde[7] have shown, can definitely influence the prothrombin time of highly diluted plasma. Most of these cases can probably be dismissed as insignificant, but authenticated cases deserve reinvestigation to determine what factor of the prothrombin complex is responsible.

The disappearance of the labile factor in plasma when stored is more easily detected in human than in dog or rabbit plasma because its concentration is relatively low. Oxidation appears to be responsible for the inactivation of the labile factor since plasma covered with a layer of mineral oil does not show nearly as rapid a decrease of this agent. Its instability appears to be associated with decalcification. Thus native hemophilic plasma shows a much slower decrease of the labile factor than oxalated or decalcified hemophilic plasma.[8] Mechanism of action and relationship of this new factor with the other clotting agents require further investigation. The complexity of the problem is plainly shown by the results in Table I.

In addition to the decrease of the labile factor it is obvious that another change occurs in stored plasma. Thus, whereas mixing fresh human plasma with an equal volume of rabbit plasma treated with tricalcium phosphate reduces the prothrombin time only to 10 seconds, the same mixture with stored instead of fresh human plasma will have a prothrombin time of 7 seconds. Something obviously happens in stored plasma which increases the activity of the labile factor. Whether this is due to the liberation of a specific substance on which the activity of the labile factor depends remains to be determined. It is interesting to speculate if this has any relation to the Ac globulin of Seegers and associates,[9] especially since they have obtained evidence that one form, the plasma Ac globulin, is converted to a more active modification which they named serum Ac globulin.[10] It should be stated that the labile factor is not Ac globulin, since agents such as tricalcium phosphate adsorb the latter but not the labile factor. It is difficult to correlate the present observations with the investigations on Ac globulin, since in those studies the influence of the labile factor is ignored and therefore not controlled. Its absence in the reaction mixture is not ruled out, however, since lung thromboplastin which was employed may contain an appreciable quantity as a contaminant.[11]

According to the most recent reports of Ware and Seegers,[12] they still hold the view that their purified prothrombin is converted to thrombin solely by thromboplastin and calcium and that their Ac globulin acts merely as an accelerator. This view is not in harmony with the earlier findings of Mertz, Seegers, and Smith[13] and of Quick[14, 15] that the conversion of prothrombin to thrombin is a stoechiometric reaction. It seems fairly certain that in this reaction the labile factor is indispensable. Owren[16] voices a similar opinion: "In 1944 and 1945 I was able to show that without factor V no thrombin is formed."

With the discovery of clotting factors other than the classic four of the Morawitz and Fuld and Spiro theory, it becomes exceedingly important to inquire how the accuracy and reliability of the prothrombin time determination are

affected. Perhaps it is best at this time, when the whole problem of coagulation seems to be entering into a new phase of development, for the sake of simplicity to view the process as involving two major groups of factors: first, the factors that constitute the prothrombin complex; second, thromboplastin and substances related to its activation. If the prothrombin time as determined by the one-stage method is normal, one can immediately conclude that the factors constituting the prothrombin complex are normal and that the defect is in the thromboplastin or in its activation. Known conditions of the latter class are hemophilia, acquired hemophilia-like disease,[17] and hemophilia-like disease of swine.[18] Hypoprothrombinemia becomes then a generic term including congenital deficiencies of components A and B and the labile factor as well as the various types of acquired conditions in which the prothrombin time is prolonged.

Neither the one-stage nor the two-stage method can be any longer considered specific for the determination of any one constituent of the prothrombin complex. It is clear that the prothrombin time of the one-stage method is a resultant of three distinct constituents, A, B, and the labile factor, and there may actually be other agents not yet recognized. The prothrombin time is therefore essentially a measure of prothrombin activity. The one-stage test has the advantage that it is performed on plasma in which physicochemical and chemical changes have been kept at a minimum. Since it has been demonstrated that the prothrombin time of native (undecalcified) and oxalated plasma is essentially the same,[6] the only major alteration of the blood in the one-stage method, namely the removal of calcium by oxalate, does not cause any fundamental disturbance. The thromboplastin employed, a saline extract of rabbit brain dehydrated with acetone, while essentially a crude preparation, has the advantage that it is almost entirely free of the labile factor and other constituents of the prothrombin complex. This is because rabbit brain can be obtained almost completely bloodless.

The determination of prothrombin activity in terms of prothrombin time of the one-stage method is valuable because evidence is accumulating both experimentally and clinically showing that it correlates with hemostatic effectiveness. Thus a prothrombin time of about 20 seconds has been found to mark the beginning of the bleeding tendency whether the deficiency is due to decrease of component B from dicumarol poisoning[19] or from congenital lack of component A.[20] It is becoming apparent that the one-stage method is a delicate and sensitive test that requires meticulous attention to details. The only satisfactory thromboplastin is rabbit brain dehydrated and prepared exactly as originally outlined by one of us (A. J. Q.) Since changes occur in plasma during storage which affect the prothrombin time, the determination should be done on plasma within one or two hours after collection.

The two-stage method of Warner, Brinkhous, and Smith likewise will require reinvestigation to determine the impact of the new prothrombin factors. The use of dehydrated lung extract as thromboplastin needs critical examination since this product is likely to contain the labile factor as a contaminant.

Likewise there is danger that even the fibrinogen employed may contain the labile factor as an impurity. This probably explains why the two-stage method failed to disclose loss of prothrombin activity in stored plasma and led Loomis and Seegers[21] into the errors of attributing the delayed prothrombin time of stored plasma to an alteration of fibrinogen and completely overlooking the true cause, namely the loss of the labile factor.

SUMMARY

The labile factor of the prothrombin complex is not adsorbed by tricalcium phosphate and similar adsorbents. It disappears from stored oxalated plasma due to destruction by oxidation and this accounts for the decrease of prothrombin activity in stored plasma.

A simple method for assaying the labile factor in plasma was developed. The plasma to be assayed is treated with tricalcium phosphate and then the amount is determined which must be added to a fixed quantity of stored human plasma to reduce the prothrombin time to an arbitrarily selected value. By this procedure it was found that rabbit plasma contains fifty times and dog plasma ten times as much labile factor as human plasma.

The prothrombin time is reduced to a markedly shorter value when the labile factor is added to stored plasma than when added to fresh plasma, thus suggesting that something is elaborated in stored plasma which increases the activity of the labile factor.

The influence of the labile factor on the accuracy of the prothrombin activity determinations and its variations in physiologic and pathologic conditions are discussed.

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BLOOD AND BONE MARROW CONCENTRATION OF ATABRINE AND ITS ROLE IN APLASTIC ANEMIA

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THE value of Atabrine in the suppression and treatment of malaria during the recent war cannot be overestimated. Despite the large number of troops to whom the drug was regularly administered, very few signs of toxicity appeared. Most prominent were the cases of Atabrine dermatitis complex. The most severe reactions caused by Atabrine therapy were in the cases of aplastic anemia reported by Custer.[1] He described fifty-seven fatal cases in troops from the Asiatic-Pacific Theater. This was the first comprehensive study indicating that Atabrine affected the hematopoietic system. Previously Shannon and co-workers[2] showed that the Atabrine concentration in leucocytes was much higher than in plasma or erythrocytes. It might be inferred from this observation that since Atabrine is concentrated in the leucocytes it might act as a toxic agent on these cells and perhaps aid in the pathogenesis of aplastic anemia.

Aplastic anemia primarily involves the bone marrow and it therefore was of interest to determine the concentration and the rapidity of elimination of Atabrine from the hematopoietic organs. In aplastic anemia the myeloid leucocytes are much more severely affected than are the lymphocytic leucocytes, and information was needed as to whether Atabrine entered both these cells in comparable concentrations.

This report presents the results of a systematic study of the Atabrine concentration in bone marrow and in various blood elements of the rabbit and chicken and the rate of excretion. These results are correlated with some observations in man.

EXPERIMENTAL

Rabbit Experiments.—The concentration of Atabrine in the bone marrow and blood of rabbits was determined at various intervals after a single injection of the drug. Rib marrow levels were taken as representative of those in flat bones, and femoral marrow was analyzed for levels in long bones. Liver, spleen, thymus, and mesenteric lymph nodes were analyzed for comparison of visceral levels with marrow levels.

Method: Each of twenty-one albino rabbits weighing about 2.5 kilograms was injected with a single intramuscular dose of 8 mg. per kilogram of Atabrine dihydrochloride. The animals were then sacrificed at intervals of from four hours to twenty-six days. Three animals were studied in each group and the results were averaged. The blood was collected in potassium oxalate and centri-

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fuged to separate the plasma and red cells from the buffy coat containing the white cells. All specimens of tissue and blood were analyzed in duplicate using Masen's photofluorometric method.[3]

Results: Table I shows the concentration of Atabrine in the tissues studied. After four hours the spleen contained 40.6 mg. per kilogram as compared with 7.7 mg. per kilogram in the liver. The bone marrow Atabrine varied between 3.1 and 5 mg. per kilogram, indicating that there was only a slight difference, if any, between the Atabrine level in flat bone marrow and in long bone marrow. The concentration of the drug in leucocytes was much greater than that found in either plasma or erythrocytes.

TABLE I. ATABRINE CONCENTRATION (MG./KG.) IN TISSUES OF RABBITS AFTER AN INTRAMUSCULAR INJECTION OF 8 MG./KG. OF ATABRINE DIHYDROCHLORIDE

	TIME AFTER INJECTION						
	4 HOURS	4 DAYS	7 DAYS	11 DAYS	13 DAYS	18 DAYS	26 DAYS
Rib marrow	5.08	0.89	0.37	Trace	0	0	0
Proximal femoral marrow	4.38	1.02	0.33	Trace	0	0	0
Distal femoral marrow	3.11	0.69	0.21	Trace	0	0	0
Plasma	0.08	Trace	0	0	0	0	0
Erythrocytes	0.27	Trace	0	0	0	0	0
Leucocytes	1.87	Trace	0	0	0	0	0
Lymph node	3.70	1.35	1.05	0.41	0.19	0.29	0.19
Thymus	2.70	0.57	0.10	0	0	0	0
Liver	7.78	1.25	0.46	0.05	0.11	0	0
Spleen	40.62	1.96	0.44	0.11	0.14	0	0

Four days after the administration of Atabrine the spleen still had the greatest concentration of drug, but this level had dropped more precipitately than had any of the others. Despite a significant level in the bone marrow, the peripheral erythrocytes, leucocytes, and plasma were practically free of Atabrine. During the second week after injection the levels in all tissues decreased rapidly. The thymus contained no Atabrine on the eleventh day, whereas the lymph nodes contained the drug as late as twenty-six days after injection.

Chicken Experiments.—Chickens are frequently utilized in studying the effects of antimalarial drugs, and therefore experiments were conducted to determine the concentration of Atabrine in the marrow and blood elements of this animal. Liver and spleen again were analyzed for comparison. Since chicken erythrocytes are nucleated, the comparative concentration of Atabrine in the leucocytes and erythrocytes was of interest.

Method: Eight Leghorn hens weighing about 1.7 kilograms each were injected with single intramuscular doses of 8 mg. per kilogram of Atabrine dihydrochloride. They were sacrificed at intervals of four hours and four, seven, and ten days. Two birds were examined at each period. The tissues were treated as described in the rabbit experiments.

Results: In general, at four hours the Atabrine concentrations in the chicken tissues were similar to those found in rabbits at a corresponding time after injection. The same relationship also existed between the various blood components, in that the erythrocyte concentration of Atabrine was higher than that of the plasma but much lower than that of the leucocytes (Table II).

TABLE II. ATABRINE CONCENTRATION (MG./KG.) IN TISSUES OF CHICKENS AFTER AN INTRAMUSCULAR INJECTION OF 8 MG./KG. OF ATABRINE DIHYDROCHLORIDE

	TIME AFTER INJECTION			
	4 HOURS	4 DAYS	7 DAYS	10 DAYS
Proximal femoral marrow	5.08	0	0	0
Distal femoral marrow	6.11	0	0	0
Plasma	0.17	0	0	0
Erythrocytes	0.91	0	0	0
Leucocytes	3.32	0	0	0
Liver	21.64	0.21	0.07	0.04
Spleen	15.82	0.10	0.04	0.05

In the case of the chickens, however, the concentration of Atabrine in the marrow was reduced to zero within the first four days, whereas this result was obtained only after thirteen days in the rabbits receiving a comparable dose of the drug. Another species difference was found in the splenic concentrations of Atabrine at four hours. In the rabbit the level in the spleen was much higher than that of the liver, whereas in the chicken the reverse was found.

Observations on Human Beings.—The purpose of these experiments was to compare the levels of Atabrine in the bone marrow with those found in the peripheral blood. By utilizing patients with either myelocytic or lymphocytic leucemia it was also determined whether Atabrine was found in similar concentrations in both lymphocytic and myelocytic leucocytes.

Method: Two male subjects, one normal and the other with a recurrence of symptoms from infection with vivax malaria, received Atabrine by mouth. Twenty-four hours after completion of a course of 2.4 Gm. of Atabrine, samples of peripheral blood and aspirated bone marrow were obtained. The bone marrow in these instances was markedly diluted with peripheral blood as compared with the bone marrow removed from rabbits after autopsy. Three additional subjects, two with myelocytic leucemia and one with lymphocytic leucemia, were given Atabrine orally for varying periods of time. The bone marrow and peripheral blood of three patients and the peripheral blood of the other two were studied.

TABLE III. BLOOD AND BONE MARROW CONCENTRATION AFTER ORAL ADMINISTRATION OF ATABRINE DIHYDROCHLORIDE TO FIVE PATIENTS*

PATIENT	CONDITION	LEUCOCYTES PER MM.^{3}	ATABRINE LEVEL (μG. × LITER)			
			PLASMA	ERYTHROCYTES	LEUCOCYTES	BONE MARROW
1	Malaria	7,000	42	–	–	836
2	Normal	5,500	77	55	–	1200
3	Chronic myelocytic leucemia	75,000	154	200	1285	2381
4	Chronic myelocytic leucemia	75,000	250	392	1589	–
5	Chronic lymphocytic leucemia	90,000	53	57	1864	–

*Doses varied so that absolute levels between patients are not comparable.

Results: As in the rabbit experiments, it was found that the bone marrow had a higher concentration of Atabrine than either the plasma or erythrocytes (Table III). The marrow concentration was as much as twenty times that of the plasma despite the fact that the marrow was mixed with peripheral blood.

The patients with leucemia, whose leucocytes were mainly myelocytic or lymphocytic cells, showed high concentrations of Atabrine in their white cells. It was thus determined that lymphocytes also had a high concentration of Atabrine similar to that found in myelocytic cells.

DISCUSSION

There have been but few reports dealing with Atabrine in the various blood elements and in bone marrow. Histologically, Martin and co-workers[4] noted deposition of the drug in various tissues although the blood and bone marrow were not specifically mentioned. Siegel and Mushett[5] reported that after large doses of Atabrine the bone marrow of rats showed an increase in the number of segmented and nonsegmented neutrophils. Reticulo-endothelial cells containing vacuoles and basophilic inclusions occasionally were found. Significant levels in the bone marrow of chickens have been noted for at least two days after a single intravenous injection of Atabrine.[6] Rather high levels were reported in rabbit marrow six days after a large oral dose.[7] The present study confirms the species difference in that Atabrine remains in the bone marrow of rabbits longer than it does in the marrow of chickens. This species difference is mentioned because of the frequent use of chickens in the study of malaria and its treatment.

This investigation revealed that although Atabrine was found in the marrow of rabbits in high concentrations, the rate of disappearance of the drug from the marrow was not markedly different from that found in other tissues. Thus eleven days after a single dose of Atabrine the bone marrow as well as other tissues showed only very small amounts of the drug present. This level soon reached zero, indicating that marrow does not retain Atabrine for long periods of time. Two weeks after completion of the Atabrine course, in one of the leucemic patients the bone marrow was free of the drug. No studies for the detection of Atabrine degradation products were undertaken.

The British Army Malaria Research Unit[8] showed that when Atabrine was given to patients with myelocytic leucemia the whole blood concentration rose as the white cell count rose. The present study, utilizing leucemic patients and analyzing the leucocytes directly, reveals that the lymphocytes as well as the myelocytic cells concentrate Atabrine in large amounts.

The question arose as to whether the ability of leucocytes to concentrate Atabrine and the inability of erythrocytes to do so was due to the fact that the leucocytes contain nuclei. It is recognized that there probably exists a fundamental difference between the nuclei of the red and white cells; nevertheless, in the chicken, in which the peripheral erythrocytes are nucleated, it was found that these cells did not have as high a concentration of Atabrine as did the white cells. From this it seems likely that in the chicken, and probably in other animals, none of the cells of the erythrocytic series take up large quantities of Atabrine and that therefore the high concentration of the drug in bone marrow is chiefly due to the presence of the drug in the myelocytic series of cells.

Although histologically the lymph node and thymus are quite similar and the concentration of Atabrine in both tissues was found to be almost the same at

TABLE IV. LACK OF CORRELATION BETWEEN ATABRINE CONCENTRATION IN BLOOD CELLS AND IN TISSUES, AND THE FATE OF THESE IN APLASTIC ANEMIA

	MYELOCYTIC LEUCOCYTES	LYMPHOCYTIC LEUCOCYTES	ERYTHROCYTES	BONE MARROW	LYMPHATIC SYSTEM
Atabrine concentration	High	High	Low	High	High
Affected in aplastic anemia	Yes	No	Yes	Yes	No

four hours, the subsequent finding was markedly different. Whereas no Atabrine was detected in the thymus at the end of eleven days, the lymph nodes retained the drug for more than twenty-six days.

An attempt has been made to correlate the present findings in animals and man. It is believed that the results obtained prove that the alleged relationship between Atabrine and aplastic anemia is not a direct one. It is not due to the fact that the drug enters the blood cells in high concentrations and, by acting locally, destroys them. Although Atabrine concentrates in both lymphocytic and myelocytic leucocytes, only the latter are affected in the disease. Although the drug does not enter the red cell series in large amounts, these cells are severely affected (Table IV). There appears to be no correlation between concentration of the drug in various blood cells and those cells affected in aplastic anemia. Because of these findings and the further fact that of the millions of people who took Atabrine only a few developed aplastic anemia, it may be presumed that the role of Atabrine in the pathogenesis of aplastic anemia falls into the idiosyncratic group of responses.

SUMMARY

After a single dose of Atabrine injected into rabbits, the concentration in the bone marrow was much higher than that in the plasma or erythrocytes. More time was required for complete excretion of the drug from the bone marrow than from the peripheral blood.

Lymphocytic and myelocytic leucocytes both contained Atabrine in relatively high concentrations. Their ability to concentrate the drug is not due to the presence of a nucleus, since the nucleated erythrocytes of the chicken do not develop as high a concentration of Atabrine as do the leucocytes.

Aspirated human sternal marrow, although greatly mixed with blood, showed a concentration of Atabrine twenty times as great as did the plasma. The mesenteric lymph nodes of the rabbit retained considerable amounts of Atabrine long after the liver and spleen were free of the drug.

There appears to be no correlation between the height of concentration of Atabrine in the various blood cells involved in aplastic anemia and the damage evident in those same cells when aplastic anemia develops. The alleged role of Atabrine in the causation of this disease would appear to be in the nature of an idiosyncrasy.

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THE EFFECT OF THE LIGATION OF THE PANCREATIC DUCTS AND OF PANCREATECTOMY AFTER DUCT LIGATION ON SERUM LIPASE

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THERE is general agreement that ligation of the pancreatic ducts in dogs leads to an immediate increase of the amylase in the blood. This unity of opinion, however, does not exist regarding the effect of tying of the ducts on the blood lipase. Cherry and Crandall[1] have found an olive oil splitting enzyme in relatively large amounts in the blood of dogs after ligation of the pancreatic ducts. The results have been confirmed in two dogs by Dozzi[2] and in cats by Roe and Goldstein[3] and Goldstein and co-workers.[9] But Popper and Sorter[4] did not observe an increase of blood lipase after the operation. Referring to the experiments of these investigators, Bauman[5] believes that elevation of blood lipase after tying the pancreatic ducts has not yet been definitely proved.

Through the work of Comfort and Osterberg[6] and Johnson and Bockus[7] the experiments of Cherry and Crandall have become the basis for the clinical use of the serum lipase test in the diagnosis of pancreatic disease. However, if Popper and Sorter's results are correct, the serum lipase test is deprived of its essential experimental confirmation.

Since we have been interested in the evaluation of this test and its experimental foundations for years, we were confronted with the following questions:

Does the serum lipase rise significantly after ligating the pancreatic ducts in dogs or not?

If the serum lipase does rise after ligation of the pancreatic ducts in dogs, do these increased amounts of serum lipase originate in the pancreas?

Is the pancreas its only source?

What is the probable mechanism for the increase?

We began our investigations with the determination of the serum lipase before and after ligation of all pancreatic ducts on a large scale in order either to confirm Cherry and Crandall's results or to disprove them.

Our findings are based on observations on dogs, chiefly female. Animals weighing from 6 to 11 kilograms were used. Those weighing about 20 pounds were preferable, since removal of 15 to 20 c.c. of blood was necessary for the chemical determinations and on some dogs many of these had to be made. Blood was withdrawn from the heart by puncture or from the jugular vein.

Dogs were fed once or twice a day on a mixture of a prepared (commercial) dog meal, evaporated milk, and canned tomatoes, with occasional supplements of ground meat when it was available.

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Anesthesia was induced with veterinary Nembutal* by the combined intravenous and intraperitoneal routes. The optimum method in our hands was to give initially an intravenous injection of about 0.2 c.c. per kilogram of body weight; after the peritoneal cavity was opened, the anesthetic solution was injected into it as needed.

Simple ligation and division of the pancreatic ducts, total pancreatectomy, partial pancreatectomy, and duct ligation followed by total pancreatectomy, after an interval of several days, have been performed in the course of our studies. In the simple ligation and division of the ducts the pancreatic lobules were separated from both the duodenum and the pancreaticoduodenal blood vessels by sharp dissection of the delicate connective tissue adherent to the pancreas and by a gentle peeling action of the bare or gauze-covered finger. The ducts were difficult to isolate, but with meticulous care few were missed. Almost always one large duct was fairly easy to identify and ligate. The other ducts, usually one, sometimes two, and rarely three, were, with exceptions, proximal to the main duct. These ducts were invariably difficult to locate as they were always short and of fine caliber. In order to diminish chances of the pancreatic juice getting into the intestine and to facilitate healing, the omentum in 12 dogs was secured to the raw surface of the pancreas or interposed between the separated pancreas and duodenum. When ligation and division of the ducts had been done previously, total pancreatectomy proved to be more difficult, due to the intense inflammatory reaction in the region of the duodenum and pancreas, than total removal of the intact normal pancreas.

LIPASE METHOD

This method for lipase determination utilizes three blanks for every determination. B_1 represents the serum blank, B_2 the olive oil blank, and B_3 the buffer blank.

B_1	B_2	B_3	TEST
2 c.c. H_2O	2 c.c. Oil emulsion	3 c.c. H_2O	2 c.c. Oil emulsion
5 c.c. Calcium acetate	5 c.c. Calcium acetate	5 c.c. Calcium acetate	5 c.c. Calcium acetate
5 c.c. Buffer	5 c.c. Buffer	5 c.c. Buffer	5 c.c. Buffer
1 c.c. Serum	1 c.c. H_2O		1 c.c. Serum

Incubate the four flasks at 37° for twenty-four hours. At the end of incubation add 10 c.c. of alcohol-ether inactivating mixture to each flask. After having added a few drops of phenolphthalein, titrate with N/20 NaOH to a definite pink. Calculation: Add B_1 to B_2 and subtract B_3. Finally, subtract this result from the test.

Example:	B_1	1.2 c.c.	B_1	1.2	Test	2.15
	B_2	0.85 c.c.	B_2	+0.85		−1.70
	B_3	0.35 c.c.		2.05		0.45 c.c.
	Test	2.15 c.c.	B_4	− .35		
				1.70		

Reagents.—

Olive Oil Emulsion: Gum acacia, 12.5 Gm.; olive oil, 50 c.c.; H_2O, 100 c.c. Pour the gum acacia and 50 c.c. of H_2O into a Waring blender and thoroughly mix. Then add the olive oil slowly and allow to mix to a pure white emulsion. Then add 50 c.c. more H_2O and mix to a uniform consistency. Store in the icebox.

*Abbott Laboratories, North Chicago, Ill.

Calcium Acetate: $Ca(C_2H_3O_2)_2 \cdot H_2O$. Calcium acetate, 20 Gm. liter of H_2O.
Barbiturate Buffer: Sodium diethylbarbiturate, 5 Gm. per liter of H_2O.
Alcohol-Ether Inactivating Mixture: Alcohol 95 per cent, 900 c.c.; ether, 100 c.c.

The results of our experiments concerning the effect of pancreatic duct ligation on serum lipase are shown in Table I and Fig. 1.

This enzyme was determined twenty-eight times in twenty dogs before the operation. The average amount of serum lipase found in these twenty dogs was 0.6 unit; the lowest value was 0.2; the highest values were 1.3 and 1.6 in one dog and 1.0, 1. and 1.2 in three other dogs; in all the other animals it was not higher than 0.8. All animals except Dog 20 showed a marked increase of the

TABLE I. THE EFFECT OF LIGATION OF THE PANCREATIC DUCTS ON SERUM LIPASE

DOG	SERUM LIPASE BEFORE DUCT LIGATION		SERUM LIPASE AFTER DUCT LIGATION (DAY)									BEYOND THE TENTH DAY
			1	2	3	5	6	7	8	9	10	
1	0.6	0.7		9.7	8.2							
2											16.3	1.2(44) 2.4(51) 9.1(89)
3	1.1				8.4							
5	1.6	1.3			2.3						2.7	1.2(41) 1.0(48)
6	0.3						9.9					10.6(16)
10	0.5	0.8	7.3	6.9								
11	0.4			7.5		6.9						
12	0.4			5.3		5.2						
13	1.0			5.0			1.2					
14	0.5				4.4			2.3				
15	0.3					7.9						
16	0.7					2.8						
17	0.6			3.5				3.5		5.8		
18	1.2			10.9				6.7		5.8		
19	0.6				2.3			1.3				
20	0.7				1.4			0.8				
21	0.3	0.2	3.6 4.1									
22	0.5	0.2	5.2		6.2							
23	0.5 0.4	0.8	4.5		7.2							
25	0.4		6.2									
26	0.2	0.4	4.8						7.8			8.6(11)

serum lipase after the ligation of the pancreatic ducts. This rise begins within twenty-four hours after the operation. We determined the serum lipase in six dogs the first day following the ligation and we found values between 3.6 and 7.3. The average amount of serum lipase in these six dogs was 0.4; the average amount twenty-four hours after the ligation was 5.1; in other words, on the average the values increased thirteen times over the initial values. On the second day after the operation the figures for serum lipase were still rising, the average being 7.0. After six or seven days the enzyme seemed to drop in some dogs, in others it appeared to rise, and in two dogs the highest values were found ten and twenty-two days after the ligation. Only Dog 20 showed an insignificant increase of serum lipase from 0.7 to 1.4 on the third day after the operation. It is possible, however, that from the fourth to the seventh day, when determinations of serum lipase were not performed, the values might have been higher.

These experiments prove definitely that after ligation of the pancreatic ducts there is almost invariably a rapid rise of the serum lipase and that this rise is of relatively long duration. However, the results do not justify the conclusion that the enzyme responsible for this rise originates in the pancreatic tissue. Other possibilities are to be considered. The pancreas may regulate

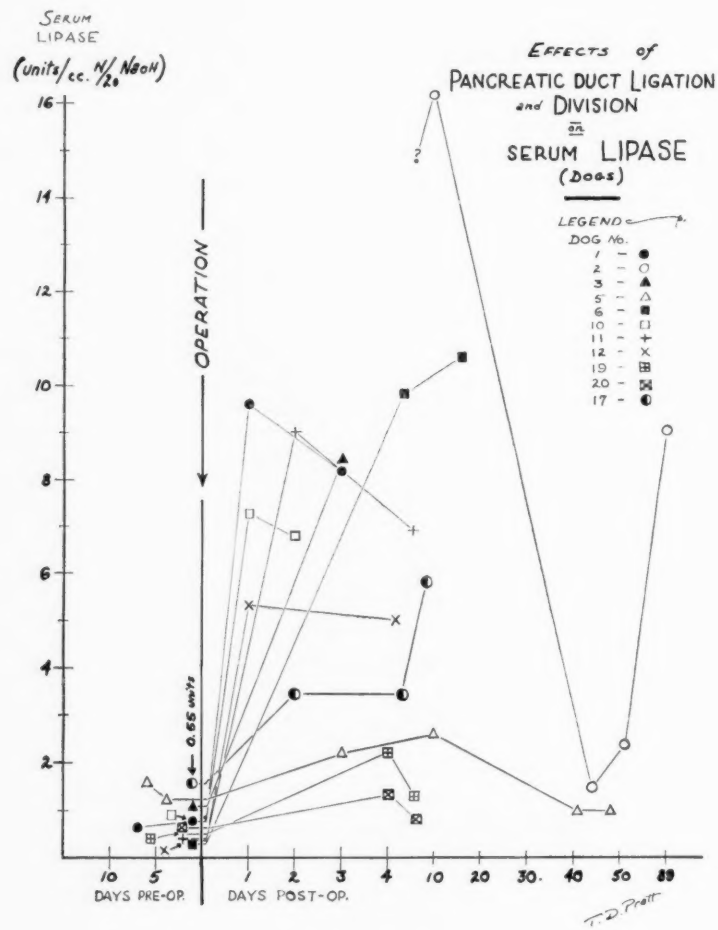

Fig. 1.

the lipase level in the blood by excretion of any excess of the enzyme which is produced in some different organ, and ligation of the ducts would lead to the elevation of the serum lipase merely by removing this regulating factor. If the serum lipase is of pancreatic origin, it should disappear after total pancreatectomy. If, however, serum lipase is derived from other sources than the pancreas and the pancreas is only the regulating factor, total removal of this gland will have the same effect as ligation of the ducts, an elevation of the serum lipase.

There were two valid methods whereby we could decide this question. We could determine the level of serum lipase after removal of the pancreas in nor-

mal dogs, or we could produce hyperlipasemia by ligating the ducts and then some days later removing the gland. Dozzi, approaching the same problem, chose the first method. Utilizing a modification of Cherry-Crandall's technique, he determined the serum lipase before and after pancreatectomy in a single dog. The values before the operation ranged from 0.1 to 1.15. On the first day after removal of the pancreas, the serum lipase was 1.05, on the second day it was 1.7, and on the fifth, shortly before the dog died, it was 0.6. The experiments of Roe and Goldstein and of Goldstein, Jacobson, Telford, and Roe performed on cats are not conclusive either, and hence one cannot base a reliable opinion upon them.

TABLE II. THE EFFECT OF PANCREATECTOMY ON SERUM LIPASE

DOG	SERUM LIPASE BEFORE PANCREATECTOMY		SERUM LIPASE AFTER PANCREATECTOMY (DAYS)				
			1	2	3	4	5
7	1.0		0.78	0.25	0.4		0.4
27	0.6	0.7	0.6	0.3	0.3	0.1	
28	0.5	0.8	0.6	0.4		0.2	

Total pancreatectomy alone was done in three dogs (Table II), while in five dogs total pancreatectomy was carried out five to nine days following duct ligation. (Table III and Fig. 2.) As can be seen from Table II, the dogs on which pancreatectomy was performed without previous duct ligation showed a steady decrease of serum lipase until death occurred five or six days after the operation. After tying all the pancreatic ducts in two dogs, the corpus pancreatis was removed, leaving the processus lienalis and processus uncinatus in situ. Thus from one-half to two-thirds of the total pancreatic tissue was extirpated. The pancreatic tissue that remained, although able to prevent diabetes, was not sufficient

TABLE III. EFFECT OF LIGATION OF THE PANCREATIC DUCTS FOLLOWED BY TOTAL PANCREATECTOMY ON SERUM LIPASE

	DOG 13	DOG 14	DOG 15	DOG 16	DOG 18
	Lipase before first operation				
	1.0	0.55	1.0	0.65	1.15
Days after ligation	*Lipase after ligation of pancreatic ducts*				
2	5.0				10.9
3		4.4			
5			7.85	2.75	
6	1.2	2.25			6.65
9					5.8
	Pancreatectomy, day after ligation:				
	6	6	5	5	9
Days after pancreatectomy	*Lipase after pancreatectomy*				
2	0.15	0.18	2.35	0.7	
3					0.1
7		0.1			
9		0.55	1.51		
16		1.1			
19			1.9		
20		1.9			
26		1.2			
	Death after pancreatectomy (day)				
	5	41	34	6	5

to cause an elevation of the serum lipase. The effect of the removal of the pancreas on the level of serum lipase where the ducts have been ligated previously is clearly indicated in Table III (Fig. 2). All five dogs showed a marked rise of the serum lipase after tying of the ducts. (These results are already included in Table I.) After removal of the pancreas there was an immediate drop of

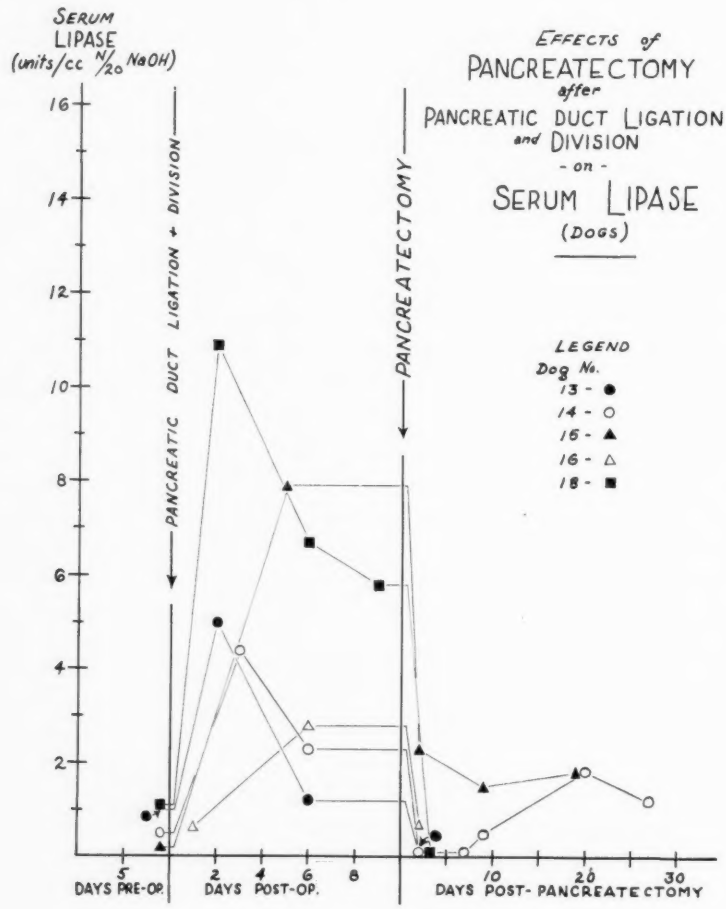

Fig. 2.

the serum lipase. In three animals (Dogs 13, 14, and 18) the values for the lipase were almost zero on the second day following the operation. In the two remaining animals (Dogs 15 and 16) there was an essential decrease of the enzyme but it was less pronounced than in the other three animals.

Dogs 13, 16, and 18 died a few days after the second operation, but Dogs 14 and 15 were still living three and four weeks after pancreatectomy. We were surprised to find rising figures for serum lipase in these two dogs. One dog showed a value of 1.9 on the nineteenth day after the operation, and the other one showed 1.9 on the twentieth and 1.2 on the twenty-sixth day after the removal of the gland.

Our experiments demonstrate that serum lipase originates at least partly in the pancreas. This is proved by these three facts: (1) Tying of the pancreatic ducts leads to an immediate rise of the serum lipase. (2) Complete pancreatectomy leads to a steady decrease of the enzyme. (3) Total removal of the pancreas at a time when the level of serum lipase is still high, as a result of ligating the ducts, leads to a rapid diminution of the enzyme almost down to zero. We are not able to explain why other authors (Popper and Sorter) did not succeed in producing elevated levels of serum lipase after ligation of the pancreatic ducts; but our findings that two to three weeks after total pancreatectomy serum lipase appears again to increase in amount, even to values higher than before operation, show that this enzyme may also be derived from extrapancreatic sources. The liver is a possible source. In studies concerning the enzymes of the bile in man, we have regularly found lipase in the contents of the gall bladder and the hepatic duct. (Unpublished data.)

The increase of serum lipase is apparently due to its absorption into the blood stream when the flow of pancreatic juice has been blocked. To substantiate this conclusion we have ligated the pancreatic ducts and drawn the tail of the pancreas through the abdominal wall, exposing the tip through the skin.

TABLE IV. THE EFFECT OF LIGATION OF THE PANCREATIC DUCTS FOLLOWED BY THE PERFORMANCE OF A PANCREATIC FISTULA ON SERUM LIPASE

	DOG 24	DOG 27	DOG 28	DOG 29
	LIPASE			
Before operation	0.4	0.9	0.3	0.4
		0.25	0.4	
After ligation of ducts, tail drawn through abdominal wall (day)				
1		2.2	3.6	
2	2.0			
3				6.2
4		3.05	6.6	
	Fistula, day after ligation			
	3	5	5	4
After fistula (day)				
1				4.0
2	1.7			
3		0.08	2.1	
6	0.5			0.3
8			0.7	
10		0.1		
13				0.6
15			0.5	
18	0.2			
32	0.4			

The serum lipase rose from 0.4 to 2.0 within forty-eight hours. We then transected the tip of the exposed gland, thereby permitting the pancreatic juice to escape. The first determination of the lipase two days after this operation showed that the lipase was already decreasing. When the fistula was well established, the serum lipase returned to normal, as is shown in Table IV. This demonstrates that the removal of the obstruction to the flow of pancreatic juice by means of a pancreatic fistula after duct ligation allows the escape of the pancreatic juice from the pancreas and therefore prevents the increase of the serum lipase level.

SUMMARY

Serum lipase is of both pancreatic and extrapancreatic origin. Its pancreatic origin is proved by the following facts: (1) Rise of serum lipase after ligation of the pancreatic ducts in all of twenty-one dogs. (2) Decrease of the serum lipase after total pancreatectomy. (3) Immediate drop of the serum lipase to almost zero in five days after total pancreatectomy, when the level for serum lipase previously has been raised by duct ligation.

The recurrence of serum lipase two to three weeks or longer after total pancreatectomy proves that there exists also an extrapancreatic source for the enzyme.

The mechanism which leads to the rise of serum lipase is absorption of the enzyme into the blood stream after blocking of the flow of the pancreatic juice. When ligation of the ducts is followed by the production of a pancreatic fistula, the elevated serum lipase drops to normal levels.

The authors wish to thank Miss Lillian Rosenberg, Miss Bernice Rubinowitch, and Miss Mary Powers for assistance in the technical determinations.

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A RESIN ARTIFICIAL KIDNEY

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WASTE products excreted by the kidneys have been removed from the body via artificial routes, such as peritoneal irrigations[1, 2] and peritoneal irrigation and gastric lavage,[3] and by in vivo dialysis.[4] The present report is concerned with preliminary observations on another technique by which such removal may be accomplished.

This artificial kidney consists of a resin bed composed of nine parts of Amberlite IR – 100 H* and one part of Deacidite.† The Amberlite is a typical cation exchange resin with exchange activity on — O^-, — COO^-, and — SO_3^- groups.[5] The Deacidite is a typical anion exchange resin with exchange activity on $\equiv N(H_3O)^+$ groups.[6]

CONSTRUCTION OF RESIN BED

A coarse mesh of each resin is obtained by sifting through a 20-mesh screen. The finer particles require a positive push and care is needed to limit pulverization. Residual fine particles are removed by thorough washes. Deacidite tends to float and remixing is necessary before packing.

The resin mixture is placed in glass columns as in Fig. 1. The column used had an inside diameter of 4 centimeters. Two lengths, 50 and 85 cm., have been tested. Rubber stoppers accommodate the inflow and outflow tubes. The outflow tube is curved down and overlaid with glass beads. During packing the glass column is rotated while maintained at a 15-degree angle in order to minimize the formation of direct channels. Additional washes encourage packing and make particle escape through the outflow negligible. The stoppered ends are sealed with shellac. A second larger glass tube (outside diameter, 7.5 cm.) is likewise sealed in place about the primary tube and accommodates a temperature control water jacket.

Autoclaving is not feasible as it tends to break the resin particles, mainly Deacidite. Sterility is attained with ethyl alcohol. The bed is thoroughly washed with distilled water. At this time the outflow water should give a negative or trace nitrogen test (no resin in solution).

OBSERVATIONS

One group of in vitro experiments included the following steps: (1) Conditioning of resin bed with Solution P‡; (2) perfusion of resin bed with Solution P containing a high concentration of urea; (3) periodic measurement of the outflow urea concentration; (4) washing with Solution P and measurement of the amount of urea recovered. The volume flow was 100 c.c. per minute.

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Technical assistant, Mr. F. A. Lyle.
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*Resinous Products Chemical Co., Philadelphia, Pa.

†Permutit Co., New York, N. Y.

‡Solution P of Odel and Ferris[7] has the following composition per liter: NaCl, 6 Gm.; KCl, 0.2 Gm.; $CaCl_2$, 0.1 Gm.; $MgCl_2$, 0.1 Gm.; NaH_2PO_4, 0.05Gm.; $NaHCO_3$, 3Gm. The glucose was lowered to 2 Gm.

Fig. 2 illustrates the results. In this experiment the 4 liters of Solution P contained 16 Gm. of urea of which 4.78 Gm. (30 per cent) were extracted. With this bed, urea concentration, and flow, the urea uptake amounted to 89 per cent at 2 liters. The wash curve mirrored the uptake curve and 4.9 Gm. of urea were washed out.

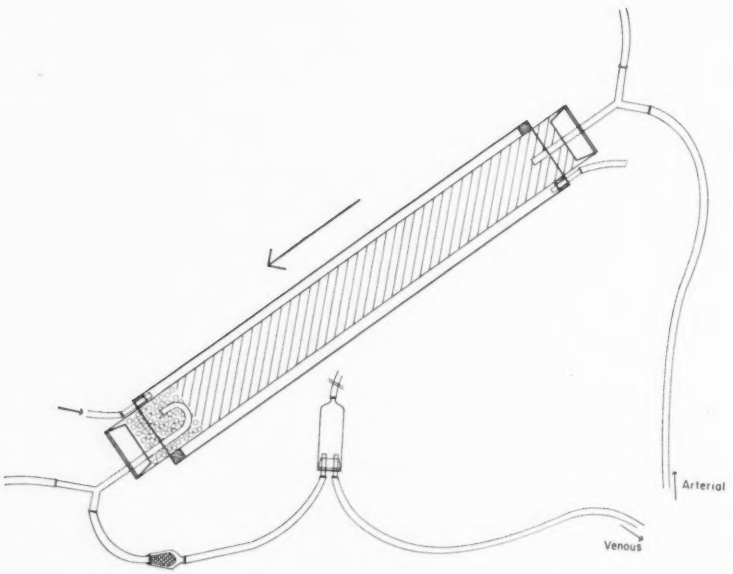

Fig. 1.—A diagramatic representation of the resin bed and connections as used in the in vivo perfusions. The description is in the text.

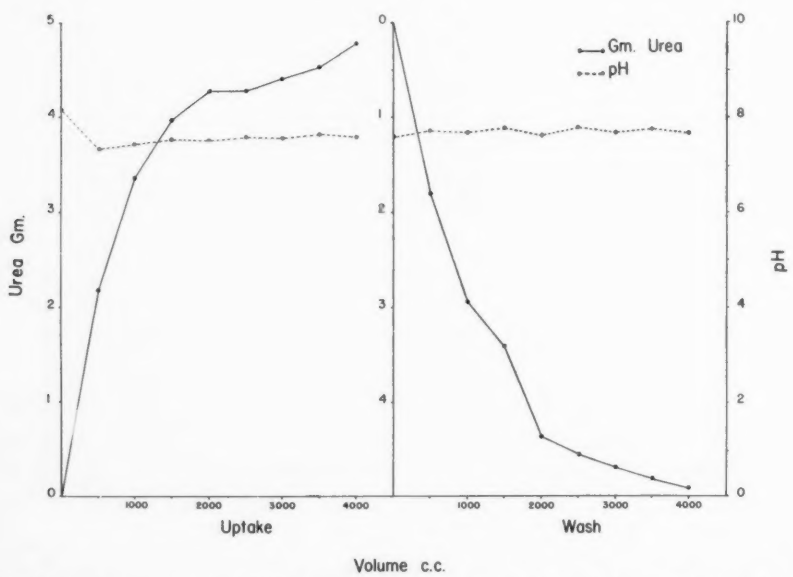

Fig. 2.—A demonstration of the urea uptake by the resin bed from Solution P containing 400 mg. per cent of urea (16 Gm. in 4 liters) and the subseqeunt urea discharge with solution P as wash. Each point represents the cumulative results up to that volume. The steady pH reading (glass electrode meter) is depicted. The following conditions pertained: resin dry weight, 600 Gm.; diameter of column, 4 cm.; length of column, 85 cm.; volume flow, 100 c.c. per minute.

Heparinized blood perfused through the bed conditioned with heparinized Solution P yielded similar results. (See Fig. 3.)

Six dogs were subjected to in vivo perfusion with this apparatus on the fourth day following bilateral nephrectomy. For these experiments the resin column was tilted to a 45 degree angle. The lowest stratum was composed only of Amberlite since the Deacidite goes into solution more readily and was removed by this Amberlite layer. The intake tube was a Y tube, one limb being

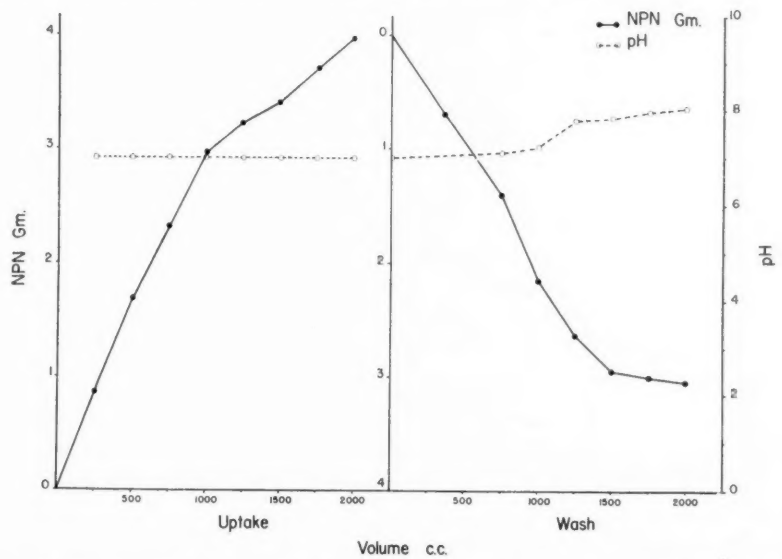

Fig. 3.—Observations similar to those in Fig. 2; heparinized blood used. Urea was added to the blood and the results were gauged as nonprotein nitrogen values. The initial nonprotein nitrogen concentration was 343 mg. per cent. The same resin bed was used and the uptake and discharge of urea were approximately twice that in Fig. 2.

connected to the femoral artery and the other to a 20-liter reservoir of heparinized Solution P. One limb of the Y outflow tube was connected to a wash collection reservoir and the other limb was connected to an inverted salvarsan graduate acting as an air trap. The air trap led to a fine mesh cloth filter and on to the femoral vein. This filter removed fibrin strands and any resin particle escaping the bed. All connections were with rubber tubing. The distance from artery to intake tube was about 60 centimeters. An interposed manometer recorded the arterial pressure. The volume flow was maintained between 75 and 100 c.c. per minute.

The resin bed was filled with dog's blood or a substitute (plasma or albumin solution) in order to prevent hypotension. Perfusion was allowed for ten minutes, then the blood residuum was collected in the air trap reservoir and the bed was washed with 1,000 to 1,500 c.c. of wash. The reservoir blood was reinfused into the vein while the bed was filling again.

Protocol, Dog 6, weight, 15 kilograms: In vivo perfusion fourth day after bilateral nephrectomy; 50 mg. heparin intravenously; wash solution: Solution P plus heparin 50 mg./per 1,000 c.c.; size of bed, 500 c.c. coarse resin; bed conditioned with 2,000 c.c. wash; temperature controlled 39° C.; number perfusions,

4, ten minutes each; flow, 70 c.c. per minute; total urea removed, 3.5 Gm. (corrected for 6 per cent blood contamination); no hemolysis, no reaction, blood pressure average 135 mm. Hg.; lived 2.5 more days, total 6.5 days.

COMMENT

Preliminary experiments show promise for the resin artificial kidney. In vitro observations demonstrate an efficient means of adjusting pH, maintaining osmolar concentration, adjusting ionic disturbances, and removing waste products. In vivo perfusions have demonstrated a minimal reaction rate and an absence of hemolysis. Waste products are removed but a more efficient means of discharging the bed of residual blood prior to the wash is needed to increase the bulk removal of wastes and to prevent an excessive fluid intake into the recipient. Observations in this direction are being conducted at present.

Reactions to the resin (mainly Deacidite) were observed earlier with insufficient wash and failure to use the final Amberlite layer to remove soluble Deacidite. The reactions included restlessness, muscular jerks, and tachypnea.

Thorough preliminary conditioning of the bed with a heparinized solution is essential to avert clotting. Metal parts are not used since they encourage clotting of heparinized blood.[4]

The efficiency of this technique requires improvement. Translation of this technique to clinical use is not indicated at present.

The resin used for the removal of wastes (Amberlite) was primarily devised for the removal of cations; it does not seem to be as efficient in the removal of nitrogenous wastes and presumably other waste products. Resins more suitable to this purpose seem highly desirable.

CONCLUSION

A rather simple technique has been described for the removal of nitrogenous waste products. In vivo experiments have demonstrated minimal reactions, no hemolysis, and a simple means of maintaining pH and osmolar concentration steady. Removal of excessive amounts of cations may be attained by varying the conditioning of the resin.

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THE RELATION OF PHENOL RETENTION TO UREMIA AND THE EFFECT OF PHTHALYLSULFATHIAZOLE AND STREPTOMYCIN ON PHENOL PRODUCTION

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FROM time to time varous urinary constituents, which are retained during renal insufficiency, have been implicated as primary factors in producing the symptom-complex known as uremia. The depression of the central nervous system seen in uremia has been attributed to the accumulation of phenols.[1, 2]

The source of the phenolic compounds which accumulate during renal insufficiency has been shown by Marenzi to be the intestinal tract.[3] He demonstrated that, while in nephrectomized animals the blood concentration of total phenols increased rapidly, in nephrectomized animals which had the entire intestinal tract simultaneously removed the blood concentration of total phenols either did not increase or increased only slightly.

It is generally thought that the phenolic compounds are produced in the intestinal tract by bacterial decomposition of ingested proteins. The specific bacteria which are responsible for the decomposition of proteins, however, are not known.

The possibility has been investigated that as a result of decreasing the bacterial population of the intestinal tract by the oral use of phthalylsulfathiazole or streptomycin the production and therefore the retention of phenols might be diminished in nephrectomized animals. From the data obtained, the importance of the retention of phenolic compounds in uremia has been evaluated.

METHODS

The animals used were healthy, adult, mongrel dogs, weighing 6 to 14 kilograms, and they were maintained before and during the experiment on a balanced diet (Ballard and Ballard). Daily control blood phenol determinations were done, usually for two days, on thirteen normal dogs. Four of these dogs then received orally a total of 0.5 Gm. per kilogram per day of phthalylsulfathiazole in divided doses for three days prior to bilateral nephrectomy and postoperatively until death. To another four animals, a total of 0.5 Gm. per day of streptomycin* was given orally in capsules in divided doses for three days prior to the operative procedure and postoperatively until death. Five control dogs which received no medication were similarly nephrectomized. The operative procedure was a one-stage bilateral nephrectomy under ether anesthesia with preanesthetic morphine and atropine. The adrenals were left intact.

Preoperative and postoperative phenol levels were done on each of the animals according to the method of Bernhart and Schneider.[4] Fresh stool specimens from each animal were studied frequently by serial dilutions of 1 Gm. wet weight of feces in Bacto-Lactose†

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†Difco Laboratories, Detroit, Mich.

broth before and during the period of drug administration to determine the coliform organism content. The greatest dilution of stool at which both acid and gas were produced in the broth was considered a measure of the number of coliform organisms in the fecal specimens. In the greater dilutions one is probably dealing with coliform organisms, but in the smaller dilutions any aerobic organisms producing gas from proteins or lactose may be included in the measurement.

All the animals were observed frequently until death, and the presence or absence of the various depressive and excitatory symptoms of uremia was recorded. The length of survival after operation was noted in all animals, and each animal was autopsied as soon after death as possible.

RESULTS

A preliminary study of the blood phenol concentrations in seventy-three determinations on twenty-four normal dogs yielded a mean value of 1.50 mg. per 100 c.c., with a range of from 0.82 to 2.30 milligrams. Only two of the

TABLE I. EFFECT OF ORAL PHTHALYLSULFATHIAZOLE AND STREPTOMYCIN ON BLOOD

DOG (WEIGHT IN KG.)	DAYS 1		2		3		4		5	
	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT (F)	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT
Control										
1 (8.7)	1.67	8	1.37	7	1.54	—	1.78	—	1.69	6
2 (9.5)	2.04	6	1.88	—	2.19	6	1.85	6	1.78	—
3 (10.7)	1.72	6	1.45	—	1.72	7	1.88	—	1.69	—
4 (7.1)	1.37	6	1.17	—	1.20	7	1.05	7	1.06	—
5 (9.5)	1.95	7	1.93	—	1.49	—	1.54	6	1.71	—
Phthalylsulfathiazole										
6 (10.2)	2.02	5	*1.88	5	1.70	—	1.84	—	2.13	2
7 (6.4)	2.07	7	*2.05	—	1.78	3	1.63	2	1.54	—
8 (11.1)	1.56	7	*1.37	—	1.15	—	1.30	2	1.33	—
9 (7.9)	1.30	7	*1.36	—	1.52	—	1.33	2	1.74	—
Streptomycin										
10 (5.7)	1.43	6	1.35	—	*1.48	—	—	<2	1.65	—
11 (6)	2.30	6	2.31	—	*1.84	—	—	<2	1.03	—
12 (7)	1.25	7	1.59	—	*1.55	—	—	<2	—	—
13 (10.7)	—	—	—	—	*1.77	—	—	<2	1.60	—

a, Lethargic; q, quiet; b, labored breathing; r, retching; v, vomiting; i, irregular heart rate; s, P, Autopsy showed death may have been due to pneumonia.
F, The approximate log of the number of organisms in 1 Gm. wet weight of feces obtained by the
*Day treatment was started.
†Died seventh day after nephrectomy. On fifth day blood sulfathiazole level was 2.9 free, 0.0 com
‡Sulfathiazole blood level: free, 4.2; combined, 0.6; total, 4.8 mg. per cent.
§Sulfathiazole blood level: free, 1.1; combined, 0.5; total, 1.6 mg. per cent.

twenty-four dogs had concentrations over 2.0 mg. per 100 cubic centimeters. This corresponds closely to normal blood phenol concentrations reported for human beings.[5]

The results in the thirteen experimental dogs are presented in Table I. In five control dogs the mean length of life after bilateral nephrectomy was ninety-nine hours, ranging from eighty-four to one hundred twenty-six hours. The four animals which received phthalylsulfathiazole lived from forty-five to one hundred sixty-nine hours after operation, with a mean of one hundred ten hours. The animals which received oral streptomycin lived only from fifty-five to eighty-one hours postoperatively, with a mean survival of sixty-eight hours.

PHENOL CONCENTRATIONS AND FECAL COLIFORM ORGANISM COUNTS IN NEPHRECTOMIZED DOGS

6			7			8			9			10		
NEPHRECTOMY														
BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	SYMPTOMS
Nephrectomized														
3.41	6	—	3.45	6	a	3.68	—	a,b, s	4.36	—	a,b r,v	4.10	— Died	a,b, s,v
2.70	—	q	2.85	—	q	3.76	—	a,r,		Died		—	—	—
2.07	—	q	3.08	—	a	3.96	—	a,b, v,r	4.57	Died 6	a,b	—	—	—
1.78	—	q		Died	P	—	—	—	—	—	—	—	—	—
2.45	—	q	4.63	—	a	5.40	6	a	6.60	Died —	a,b, r	—	—	—
Treated Nephrectomized														
3.11	—	a,x	3.65	2	a	4.99	—	a,b, i,g a,s	5.18	— Died	a,b, t,r a,s,t,	7.35	Died‡ 2	a,b,t, s,v
2.29	2	— a	3.11 4.83	— —	— a	3.38	—		3.70	—	r,v	—	—	—
2.20	—			Died	§	—	—	—	—	—	—	—	—	—
2.32	—	a	4.60	—	a	5.14	3	a	6.30	—	a,s	†	—	—
Treated Nephrectomized														
1.30	—	q	1.54	Died —	a	—	—	—	—	—	—	—	—	—
1.54	—	—	1.48	2	a	2.09	Died —	—	—	—	—	—	—	—
1.60	—	—	2.23	2	a		Died		—	—	—	—	—	—
1.96	—	—	2.14	<2	a	2.14	Died —	a,r	—	—	—	—	—	—

watery stools; g, staggering; x, abnormally belligerent (snapping, growling); t, myoclonic twitching.

effect of serial dilutions on gas and acid production in lactose broth.

bined, 2.9 mg. per cent total.

The blood phenol concentrations of the control animals taken the day of or the day prior to death were from 1.78 to 6.60 mg. per 100 c.c., with a mean of 4.16 milligrams. The animals receiving phthalylsulfathiazole had blood phenol concentrations at death of from 3.70 to 7.35 mg. per 100 c.c., with a mean of 5.54 milligrams. The animals given streptomycin died with blood phenol concentrations ranging from 1.54 to 2.23 mg. per 100 c.c., with a mean of 2.00 milligrams. Drug administration caused no significant change in the blood phenol concentrations in unoperated animals.

No animals showed evidence of predominantly increased nervous system irritability, although occasional coarse muscular twitchings were seen. All showed progressive nervous system depression terminating in death. Almost all of the animals showed severe anorexia, and they retched and vomited during the last forty-eight hours of life; one control animal had diarrhea. There was no significant difference in the uremic signs in the three groups. Some which received streptomycin showed earlier appearance of uremic signs than did the animals in the other two groups. The depth of depression was not always directly proportional to the concentration of blood phenols in the control and phthalylsulfathiazole groups.

Stool bacteriologic examinations revealed that within forty-eight hours of the start of oral phthalylsulfathiazole or streptomycin administration the coliform counts decreased from 10^6 to 10^7 organisms per gram of wet stool to 10^2 or less, and this level was maintained until the death of the animal. In general, streptomycin reduced the coliform counts somewhat more than did the sulfonamide.

Autopsy examinations revealed nothing of important significance.

DISCUSSION

Relationship Between Blood Phenol Concentration and Uremic Depression.—Becher[1] and Harrison and Mason[2] demonstrated experimentally that signs of depression in uremia are accompanied by an elevation of the blood phenol concentration. This was confirmed clinically by Dickes[17] and Roen,[18] among others, who reported that the correlation between elevated blood phenol concentration and the signs of narcosis was much closer than between the same signs and any other known chemical abnormality in uremia. They therefore concluded that there was an etiologic relationship between the two. This argument was strengthened by the fact that the clinical picture of phenol intoxication resembles the depressive aspects of uremia. On the other hand, Nesbit, Burke, and Olsen[5] in studying patients with uremia due to postrenal obstruction concluded that although the blood phenol concentration is elevated in uremia, uremic symptoms, including those of narcosis, are not always related to an elevated phenol concentration. They found no parallelism between the concentration of blood phenols and the intensity of symptoms and signs.

In our animals the administration of streptomycin effectively maintained the blood phenol concentrations at or near normal figures without altering the symptomatology. There appeared to be an earlier onset of symptoms and

earlier death in the streptomycin group. In addition, although all control animals and those receiving phthalylsulfathiazole had increased blood phenol concentrations, there was no striking correlation between the severity of narcosis and the concentration of blood phenol. In several instances concentrations over 3.5 mg. per 100 c.c. were attended by mild depression only, while in others blood phenol concentrations lower than this were associated with severe stupor. These findings make it unlikely that blood phenols are etiologically related to the symptoms of depression in uremia. There must be some other retained urinary constituents which produce these symptoms.

The survival times in the control and phthalylsulfathiazole groups agree with those previously reported. Rodbard[6] reported a survival after bilateral nephrectomy ranging from sixty to one hundred thirty hours, with a mean of eighty-five hours. Harrison and Mason[2] reported a survival in dogs of from eighty-five to one hundred twenty hours after the animals became completely anuric. The survival time of the group which received streptomycin was considerably less than that of the other two groups, but because of the small number of animals in each group there is some doubt that this difference is significant. The explanation for this is not known, since it is known that streptomycin is not absorbed from the intestinal tract in appreciable amounts. However, it is possible that degradation or conversion products of streptomycin, which would not be measured with the usual methods if they were not bacteriostatic, may accumulate and account for the earlier death.

Since the animals receiving streptomycin died earlier than those in the other two groups, it might be argued that the lower phenol concentrations in the streptomycin group could be attributed to this fact. However, the mean blood phenol concentration of the control group forty-eight hours postoperatively was 3.50 mg. per 100 c.c., and the mean forty-eight-hour blood phenol concentration of the phthalylsulfathiazole group was 4.05 mg. per 100 cubic centimeters. At a corresponding time the mean blood phenol concentration of the animals receiving streptomycin was 1.85 mg. per 100 cubic centimeters. Streptomycin therefore must effect the intestinal organisms responsible for the major portion of phenol production.

Phenol Production by Bacterial Action and the Effect of Chemotherapeutic Agents.—Phthalylsulfathiazole[7, 8] and streptomycin[9, 10] taken orally are poorly absorbed from the intestinal tract, and thus they exert nearly all of their antibacterial effect within the lumen of the intestine.

Poth and Ross[8] reported that phthalylsulfathiazole markedly depressed coliform and vegetative clostridial counts in human feces, but it had no effect on spores or on the *Streptococcus faecalis*. On the other hand, Miller[11] has shown in the rat that although coliform organisms are decreased by this drug, there is no significant change in the number of aerobic or total anaerobic organisms or in the number of spores in the feces. Segel, Schweinburg, and Fine[13] reported that this drug decreases gas formation in obstructed intestinal loops in cats by inhibiting the action of the coli-aerogenes-proteus group and certain of the clostridia. In our animals the coliform group was markedly

decreased in number by phthalylsulfathiazole administration; we did not study its effect on other organisms. However this drug produced no change in the course of the uremia nor did it diminish the usual increase in the blood phenol concentration. Apparently phthalylsulfathiazole does not act on those organisms in the intestine which are responsible for the production of phenols. Our results agree with those of Banker and Schmidt[12] who reported that the administration of phythalylsulfathiazole to normal dogs resulted in no change in the concentration of phenols in the urine in spite of the almost complete inhibition of fecal coliform organisms. They concluded that the phenol-producing organisms were not affected.

Oral streptomycin also markedly diminishes fecal coliform organisms.[14] Zintel[15] gave 1 Gm. of streptomycin per day orally in divided doses to fifteen patients and noted a marked depression of fecal coliform and streptococcal organisms and a moderate diminution of clostridia. In comparison, succinylsulfathiazole, a drug similar in its action to phthalylsulfathiazole, diminished fecal coliform organisms moderately and clostridia and *Str. faecalis* not at all. Smith and Robinson,[16] using an oral dose of 30,000 to 300,000 units per kilogram in mice, found that streptomycin eliminated all gram-negative organisms and most gram-positive ones from the feces, leaving only a small number of gram-positive spore-forming organisms. We found a marked inhibition of coliform organisms by oral streptomycin, but we did not study its effect on other fecal organisms.

The specific identity of the organisms responsible for phenol production is not known. In considering the possibilities, it appears that the organisms probably would belong to that group manifesting great hydrolytic activity toward proteins, and that they also would be sulfonamide resistant and usually streptomycin susceptible. Of the organisms usually found in feces, the ones with the greatest hydrolytic activity toward proteins[19] are *Clostridium sporogenes* and *Proteus vulgaris*. Of these two, the former is sulfonamide and streptomycin resistant while the latter is sulfonamide resistant but is usually sensitive to streptomycin. There is some basis then for the hypothesis that a small portion of phenol production may be attributed to *Cl. sporogenes* and that *P. vulgaris* is principally responsible for phenol production. It is also possible that the production of phenols requires the integration of the metabolic activities of more than one organism in the production of one or more precursors of phenols.

SUMMARY

Blood phenol determinations were done on three groups of dogs. After a control period, usually two days, phthalylsulfathiazole was administered orally to one group of four dogs in divided doses totalling 0.5 Gm. per kilogram per day until death. In the same way streptomycin was administered orally to one group of four dogs in divided doses totalling 0.5 Gm. per day until death. The third group of five animals served as a control. A one-stage bilateral nephrectomy was done on the experimental groups three days after the drug administration was started and five days after the start of the experiment in

the control group. Fresh stool specimens from each animal were studied frequently by serial dilutions in Bacto-Lactose broth to determine the coliform organism content.

The control mean blood phenol concentration was 1.50 mg. per 100 c.c., with a range of 0.82 to 2.30 milligrams. The control group died with a mean blood phenol concentration of 4.16 mg. per 100 c.c., with a range of 1.78 to 6.60 milligrams. The phthalylsulfathiazole group died with a mean phenol concentration of 5.54 mg. per 100 c.c., with a range of 3.70 to 7.35 milligrams. The streptomycin group died with a mean phenol concentration of 2.00 mg. per 100 c.c., with a range of 1.54 to 2.23 milligrams.

None of the animals showed evidence of predominantly increased nervous system irritability, although occasional coarse muscular twitchings were seen. All animals showed progessive nervous system depression terminating in death. There was no striking correlation between the blood phenol concentrations and the depressive signs of uremia.

It is concluded that the retention of phenolic compounds is of little or no significance in producing the signs of nervous system depression seen in uremia.

Within forty-eight hours of the start of phthalylsulfathiazole and streptomycin administration, the fecal coliform counts decreased from 10^6 to 10^7 organisms per gram of wet stool to 10^2 or less, and this level was maintained until the animals' deaths.

It is concluded that the predominant organism responsible for phenol production in the intestinal tract does not belong to the coliform group and that it is not inhibited by phthalylsulfathiazole but is inhibited by streptomycin. It is suggested that this organism is probably *P. vulgaris*.

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MECHANISMS OF DESOXYCORTICOSTERONE ACTION

I. Relation of Fluid Intake to Blood Pressure

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ESSENTIAL hypertension is characterized by a type of sustained blood pressure elevation in which the usual mechanisms for counteracting rises in pressure do not appear to have been brought into operation.[1] Preliminary results of investigations now in progress suggested that desoxycorticosterone may possess an ability, perhaps to a unique degree, to elevate mean pressure without production of secondary cardiac slowing or pulse pressure increase. Consequently it seemed desirable to study the mechanisms by which this drug affects the height of the blood pressure level.

The relatively rapid return to normal pressure levels following adequate desoxycorticosterone acetate (DCA) therapy in Addisonian crisis is usually considered to reflect the restoration of an adequate blood volume consequent upon correction of renal sodium and water loss.[2] On the other hand, the hypertension which may develop subsequent to DCA overdosage both in patients with Addison's disease[3] and in normal subjects[4] is not explicable on the basis of abnormal sodium retention[4, 5] or elevated plasma volume,[4, 6] and with few exceptions[7] requires a period of weeks to months for its induction.[2, 4, 8] These considerations, coupled with the absence of any conclusive demonstration of a contractile action on vascular musculature, suggest that DCA-induced hypertension may constitute a reaction to some more direct effect of the compound which precedes the pressure elevation in time.

Knowledge of the more immediate action of DCA is concerned largely with its influence on water and electrolyte balance. Although these effects are considered most frequently in relation to tubular reabsorption of sodium, their clinical expression is perhaps more evident in terms of over-all fluid exchange. Cortical insufficiency is accompanied by a diminished capacity for water excretion,[9] while excessive DCA administration is followed by a diabetes insipidus-like syndrome.[10] Since it appeared possible that the development of elevated blood pressure might be indirectly related to the profound influence of the salt-retaining steroids on fluid exchange, it was decided to study the temporal course of the two phenomena in a quantitative manner.

EXPERIMENTAL PROCEDURES AND RESULTS

The experimental animals for the first group of studies consisted of twenty rats of the Sprague-Dawley strain, weighing approximately 65 grams. All animals were kept in separate cages and were fed on Purina laboratory chow. Sodium chloride solution, 0.86 per cent, was substituted for drinking water. Consumption of food and fluid was unrestricted. Fluid intake was measured daily, and weight, weekly. The mean daily fluid intake per gram of body weight

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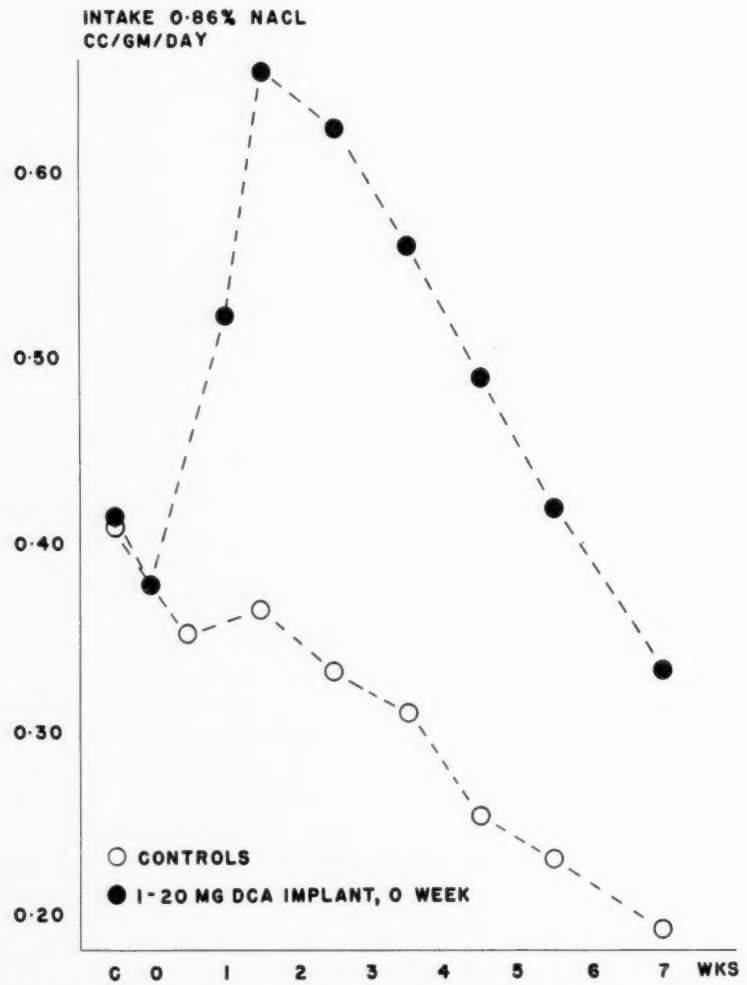

Fig. 1.—Alterations in fluid intake following DCA implantation in young rats.

was calculated for each animal once a week by dividing the average daily intake by the mean weight for the week.

At the end of the first week the animals were paired as closely as possible according to weight, unit fluid intake, and sex. Single 20 mg. DCA pellets with an absorption time of four to six months were implanted beneath the skin of the back of each of the ten animals comprising the test group, using ether anesthesia. The animals of the control group were sham operated. Measurements of weight and intake were continued as described for a total of eight weeks.

The daily fluid intake of the control group per gram of body weight declined with age in linear fashion as illustrated in Fig. 1. The test group, following implantation, manifested an abrupt and marked increase in intake, maximal within ten days, to a value twice that of the control group.

When this maximum increase had been attained, the unit fluid intake thereafter decreased with age in the test group also. However, the rate of de-

cline exceeded that displayed by the control animals, so that with the passage of time the unit intake of the test group approached that of the control group despite the marked rise which had followed DCA implantation.

Studies of the relation of these changes to blood pressure and adrenal function were carried out on a second test group of thirty rats. In addition to weight and intake determinations as previously described, the blood pressure of each animal was measured weekly by an adaptation of the tail method.[11] In order to secure the greatest degree of reproducibility it was found desirable to preheat the animals for twenty minutes at approximately 40° C. and to make a series of ten readings at each determination, the first five being discarded. Under these circumstances a group of eighteen control animals followed over a three-month period manifested blood pressure levels which never exceeded 135 mm. Hg.

Following a two-week control period the test animals were divided into two groups. The twelve rats in the first group were each implanted subcutaneously with ten 20 mg. DCA pellets. Six of the animals in this group were adrenalectomized; the remainder were sham operated. The eighteen rats comprising the second group were implanted with single 20 mg. DCA pellets. Adrenalectomies were performed on twelve animals and sham operations on the remaining six. The sexes were represented equally in each subgroup. Measurements of weight, fluid exchange, and blood pressure were continued as described for a total of twelve weeks.

Influence of Dosage.—Following implantation all animals manifested the abrupt rise and secondary regression in intake previously described. However, the maximum increase in intake was approximately three times as great at the higher dosage level. At the maximal point, which was reached in approximately ten days, the animals implanted with ten pellets were drinking more than their body weight of saline daily. They appeared to spend the greater part of the time lying on their backs, the front paws clasped about the drinking tube from which they would drink rapidly and almost without interruption for minutes on end. During the period which comprised roughly the second week following implantation, the animals appeared nervous and irritable and displayed muscular weakness and bouts of transitory paralysis during which the hind limbs were dragged. These phenomena resembled certain of the toxic manifestations which occur in man[2] and in the dog[12] consequent upon depression of serum potassium levels.

When the point of maximum intake had been passed and the period of regression toward lower levels of fluid exchange had set in, the general condition of the animals spontaneously improved. They became tractable to handling once more and all evidences of muscular weakness disappeared.

At both dosage levels the blood pressure rose slowly and in reasonably linear fashion throughout the period of observation. However, the slope of the pressure curve at the higher dosage was approximately twice as great as that at the lower level. The pressure of the animals implanted with ten pellets averaged 200 mm. Hg at the end of twelve weeks as compared with a mean of 160 mm. in those bearing a single pellet.

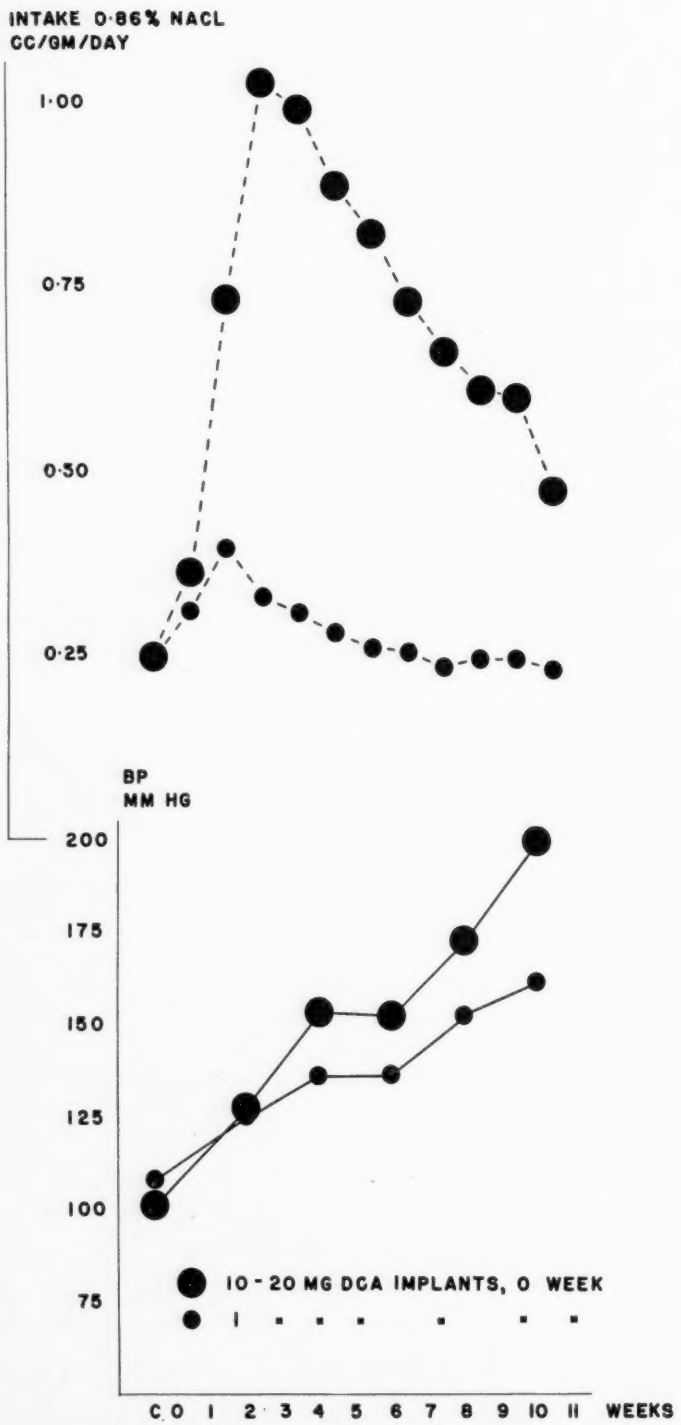

Fig. 2.—Relation of fluid intake and blood pressure following DCA implantation in young rats at two dosage levels.

When the temporal relation of intake to blood pressure was studied, as illustrated in Fig. 2, it became evident that the earliest effect at both dosage levels was the change in fluid intake, which had reached its crest and was declining before the blood pressure became elevated significantly above normal

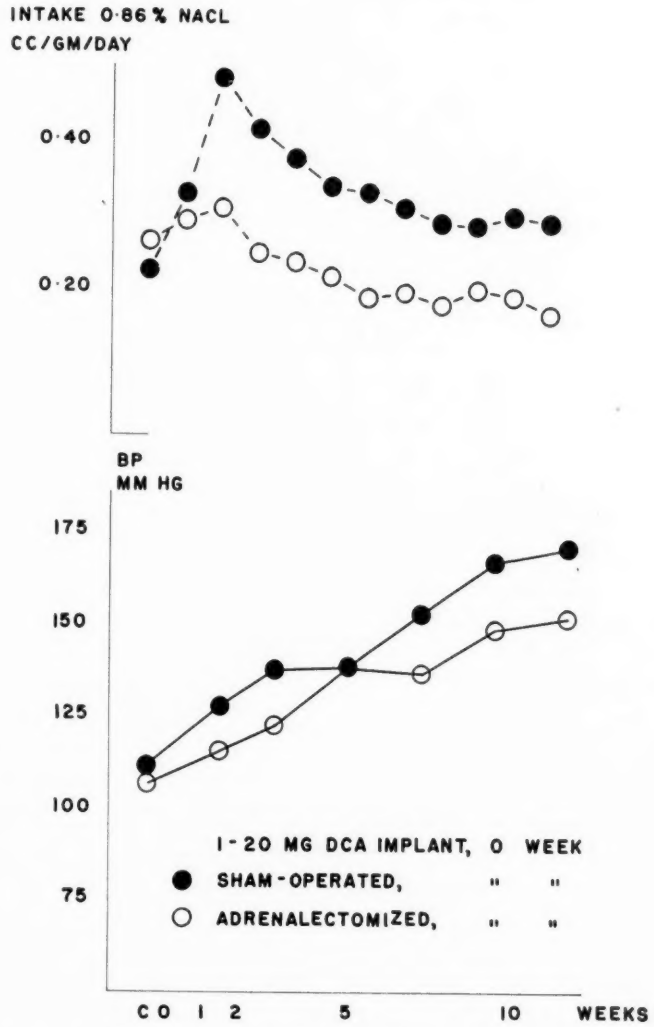

Fig. 3.—Comparison of the responses of adrenalectomized and sham-operated rats to DCA implantation at low dosage.

limits. It also appeared that the intensity of hypertension which subsequently developed was proportional to the maximal increase in fluid exchange, while its rate of development was reciprocally related to the secondary regression in intake.

Influence of Adrenalectomy.—The effect of adrenal removal on DCA action was studied because of observations that adrenalectomized dogs[13] and patients with Addison's disease[14] seemed more susceptible to overdosage than did normal

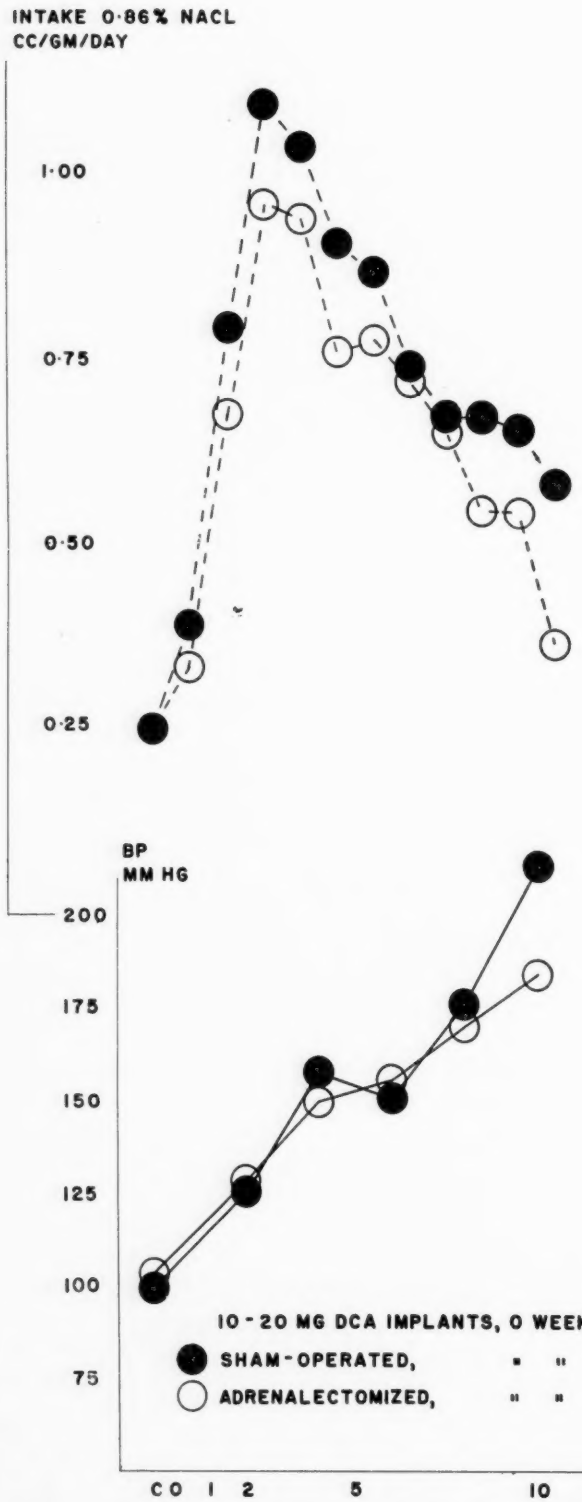

Fig. 4.—Comparison of the responses of adrenalectomized and sham-operated rats to DCA implantation at high dosage.

subjects. This apparent hypersusceptibility has been attributed to a lack of antagonistic substances ordinarily secreted by the adrenal.[14]

The implantation of single 20 mg. pellets into adrenalectomized rats was followed by a smaller rise in fluid intake and a lower grade of hypertension than that produced in sham-operated animals, as illustrated in Fig. 3. These differences became less evident with an increase in dosage, as shown in Fig. 4.

SUMMARY AND CONCLUSIONS

The immediate effect of DCA implantation in young rats maintained on isotonic saline solution was a rise in fluid intake. The more delayed responses to the drug included a secondary regression of intake values toward control levels and the reciprocal development of hypertension.

The degree of hypertension which developed appeared proportional to the dosage of the drug, the maximal rise in fluid intake, and the subsequent rate of decline in intake.

No evidence was found that adrenalectomy sensitized the test animals to the actions of desoxycorticosterone.

The possibility is suggested that the hypertension induced by DCA overdosage may not represent a direct action but may be a compensatory mechanism for overcoming distortions in fluid and electrolyte balance produced by the drug.

We are indebted to Dr. E. Oppenheimer, of Ciba Pharmaceutical Products, Inc., Summit, N. J., both for generosity in supplying desoxycorticosterone and for many helpful suggestions and criticisms throughout these studies.

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ACTIVITY OF MICROBIAL ANIMAL PROTEIN FACTOR CONCENTRATES IN PERNICIOUS ANEMIA

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THE presence of an unidentified Factor X in concentrated antipernicious anemia liver extracts was shown in experiments with rats which received a purified diet containing hot-alcohol-extracted casein.[1] Attention has been drawn[2] to the similarity of Factor X to the cow manure factor[3, 4] which promotes growth in chicks and which in turn appears to be similar to the animal protein factor (APF) needed for hatchability of hens' eggs[5] and for chick growth.[3]

In the present investigation it was found that a nonmotile, rod-shaped organism from hen feces, when grown aerobically on simplified media containing no appreciable quantities of animal protein factor, could produce this factor,* as indicated by assay with chicks on diets containing all the known B-complex factors together with high levels of soybean meal or of alcohol-extracted casein. Concentrates were prepared from the growth medium, and by means of the chick assay their potency was standardized against refined liver extract, 10 U.S.P. units per milliliter, which produced a similar growth response in chicks. Concentrate I appeared to have between 50 and 100 per cent of the activity of the 10-unit liver extract when injected into chicks. It was prepared by preliminary clarification followed by precipitation with ammonium sulfate. It contained only approximately 0.5 μg of folic acid per milliliter, as indicated by *Streptococcus faecalis R* assay, and the value was not increased by treatment with a chicken pancreas "conjugase" preparation. Concentrate I was administered parenterally to a patient with pernicious anemia.

E. G., a 90-year-old white woman, entered the hospital with an erythrocyte count of 1,290,000 per c.mm.; hemoglobin, 4.3 Gm. per 100 ml.; leucocyte count, 3,050 per c.mm.; hematocrit, 13 per cent; mean corpuscular volume, 100.5 c. μ; mean corpuscular hemoglobin, 33.3 $\mu\mu$g; mean corpuscular hemoglobin concentration, 33 per cent. The differential blood count, in per cent, was: neutrophiles, 75; eosinophiles, 1.0; lymphocytes, 20.5; monocytes, 3.0. The neutrophiles had highly segmented nuclei and were large cells. The erythrocytes showed marked variation in size and shape and appeared macrocytic. The platelets were decreased in number.

Gastric analysis showed absence of free hydrochloric acid after stimulation with histamine. Sternal marrow aspiration revealed a marrow with increased cellularity, a relative and absolute increase in nucleated red cells, and a megaloblast content of 24.4 per cent.

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*The isolation of the active organism and the microbiologic production of animal protein factor concentrates were under the direction of Dr. Milton A. Petty, Lederle Laboratories Division, American Cyanamid Company. These studies will be the subject of a separate publication at a later date.

TABLE I. PATIENT E. G., GIVEN APF CONCENTRATE I; CITRATED WHOLE BLOOD (500 ML.) ADMINISTERED ON SECOND DAY OF TREATMENT

DAY OF TREATMENT	R.B.C. (MILLIONS PER C.MM.)	HB. (GM. PER 100 ML.)	W.B.C. (PER C.MM.)	RETICULOCYTES (%)	HEMATOCRIT (%)	M.C.V. (C. μ)	M.C.H. ($\mu\mu$G)	M.C.H.C. (%)	INTRAMUSCULAR THERAPY
									APF concentrate I (ml.)
0	1.29	4.3	3,050	1.3	13	101	33	33	1.0
1	1.07	3.7	4,550	0.8					1.0
2	0.99	3.5	6,550	0.8					1.0
3	1.86	5.7	4,900	0.8					1.0
4	1.63	5.0	3,850	1.6					1.0
5	1.95	6.0	4,550	2.7					1.0
6	1.90	6.3	5,900	4.1					1.0
7	1.84	6.3	4,450	6.8					1.0
8	1.41	5.1	8,050	11.2					1.0
9	1.98	6.3	12,400	18.0					--
10	1.88	6.0	6,900	**20.6**					--
11	1.94	7.2	----	17.8					--
12	1.90	6.1	6,100	15.1					--
13	2.38	7.5	7,300	12.6					--
14	2.39	7.8	7,300	10.1					--
15	2.01	6.9	4,500	6.9					Purified liver extract* 10 u./ml. (ml.)
16	2.43	7.7	6,100	5.1					
17	2.37	7.9	5,450	4.8	23	97	33	34	
18				1.2					1.0
19	1.93	8.2	6,900	2.2					1.0
20	2.10	8.1	7,850	2.4					1.0
21	2.12	7.3	5,800	2.1					1.0
22	2.37	8.2	5,000	3.3					1.0
23	2.29	7.8	4,300	4.2					1.0
24	2.38	7.8	3,000	**6.0**					1.0
25				4.7					1.0
26	2.58	8.6	4,000	4.5					1.0
27	2.90	8.9	4,050	4.5					--
28	2.79	8.9	3,900	3.5					--
29	2.73	9.2	4,450	2.5					--
30	2.98	9.2	3,800	1.8	30	101	31	31	3.0
31	2.90	9.3	5,250	1.9					--

*Lederle.

Animal protein factor concentrate I was given in daily intramuscular doses of 1.0 ml. (Table I) for nine doses. The patient was very ill, with severe nausea and fecal vomiting. X-ray studies of the abdomen showed multiple distended loops of small intestine. A Miller-Abbott tube was inserted, but after the third dose of animal protein factor concentrate the nausea and vomiting ceased and did not reappear. A single blood transfusion of citrated blood (500 ml.) was administered when the erythrocyte count fell to 990,000 per c.mm. on the third hospital day. The patient made a rapid symptomatic recovery, becoming more alert and active, and appetite returned. Thereafter the clinical course was uneventful.

A peak of 20.6 per cent reticulocytes occurred on the tenth day after the start of treatment with animal protein factor concentrate. This was followed by a prompt increase in the numbers of erythrocytes, leucocytes, and platelets, and in the hemoglobin level (Table I). The reticulocyte response was more delayed than the average response resulting from effective intramuscular therapy with liver extracts, but the level reached by the reticulocytes was nearly that of the theoretic maximal.[6]

A course of liver extract was started eighteen days after the beginning of therapy with animal protein factor concentrate. The patient received nine daily intramuscular doses

of 1.0 ml. of purified liver extract (Lederle), each 1.0 ml. containing 10 antipernicious anemia units. On the sixth day of treatment with liver extract there was a second, small reticulocyte peak of 6.0 per cent. The patient continued to improve.

Animal protein factor concentrate II was prepared by clarification without precipitation with ammonium sulfate. It had between 25 and 40 per cent of the activity of 10-unit liver extract when fed to or injected into chicks. It contained about 0.02 μg of folic acid per milliliter, as indicated by assay with *Str. faecalis* R, and the value was not increased by treatment with "conjugase." The concentrate also was assayed for total pteroylglutamic acid with chicks and was found to contain not more than 5 μg per milliliter. In another hospital this concentrate was administered parenterally to a patient with pernicious anemia, and the findings were placed at our disposal.

E. N., an 80-year-old white woman, entered the hospital with an erythrocyte count of 1,890,000 per c.mm.; hemoglobin, 7.7 Gm. per 100 ml.; leucocyte count, 2,400 per c.mm.;

TABLE II. PATIENT E. N. GIVEN APF CONCENTRATE II

DAY OF TREATMENT	R.B.C. (MILLIONS PER C.MM.)	HB. (GM. PER 100 ML.)	W.B.C. (PER C.MM.)	RETICULOCYTES (%)	HEMATOCRIT (%)	M.C.V. (C. μ)	M.C.H. ($\mu\mu$G)	M.C.H.C. (%)	INTRAMUSCULAR THERAPY
									APF concentrate II (ml.)
−4	1.89	7.7	2,400	0.4	24	127	41	33	
−2	1.80	7.7	3,700	1.0	23	129	43	33	
−1				0.4					
0	1.87	7.8	2,700	0.9	24	128	42	33	1.0
1				2.4					1.0
2	1.98	8.2	3,900	1.9	26	133	41	31	1.0
3				1.9					1.0
4	2.08	7.9	6,000	3.7	24	116	38	33	1.0
5				6.2					1.0
6	2.17	8.1	4,400	6.4	26	120	37	32	1.0
7				9.7					1.0
8	2.28	8.2	----	6.1	26	113	36	32	1.0
9				6.0					1.0
10	2.63	9.0	5,600	3.5	30	114	30	30	1.0
11				1.8					1.0
12									1.0
13	3.0	9.5	6,900		31	105	32	30	1.0
14									1.0
15									1.0
16									1.0
17	2.81	8.9	6,900		31	109	32	29	1.0
18									1.0
19	3.18	9.3	7,300		32	99	29	29	1.0
20									1.0
21									1.0
22									1.0
23	3.33	9.8	6,300		34	100	29	29	1.0
24									1.0
25									1.0
26									1.0
27									1.0
28									1.0
29									1.0
30	3.38	10.8	11,600	1.0	35	102	31	32	1.0

hematocrit, 24 per cent; mean corpuscular volume, 127 c.μ; mean corpuscular hemoglobin, 41 $\mu\mu$g; mean corpuscular hemoglobin concentration, 33 per cent.

Gastric analysis showed absence of free hydrochloric acid after stimulation with histamine. Sternal marrow aspiration revealed megaloblastic hyperplasia.

The presenting complaint was inability to walk. The patient had a cloudy sensorium. There was loss of vibratory sensation in the lower extremities.

Animal protein factor concentrate II was given in daily intramuscular doses of 1.0 ml. (Table II) for twenty-four days. A reticulocyte peak of 9.7 per cent occurred on the seventh day after the beginning of treatment. There was a prompt increase in the number of erythrocytes and leucocytes and in the hemoglobin level (Table II).

Coincident with the hematologic response there was clinical improvement. The patient became better oriented and there was a return of appetite. The observers considered the clinical improvement to be moderately pronounced.

After thirty days of treatment the erythrocyte count had risen to 3,380,000 per c.mm., and hemoglobin to 10.8 Gm. per 100 ml.; the leucocyte count was 11,600 per cubic millimeter. There was a decrease in the mean corpuscular volume and in the mean corpuscular hemoglobin.

DISCUSSION

The results indicate that the concentrates of material produced microbiologically, and found to exert animal protein factor activity in chicks, also were active in inducing an hematopoietic response in pernicious anemia.

It is not possible to conclude that the hepatic and bacterial substances responsible for the activity in chicks are identical with the classic antipernicious anemia factor, since the so-called refined liver extracts used, as well as the bacterial concentrates, are relatively crude materials. The nearly maximal reticulocyte response to concentrate I occurred on the tenth day, while the patient given the weaker concentrate (II) had a submaximal reticulocyte response on the seventh day. With liver extracts of such potency (10 and 2.5 to 4 units per cubic centimeter respectively) a peak reticulocyte response would have been expected earlier than occurred in the first case. However the apparent delay in the reticulocyte response may have been due to the very critical state and the advanced age (90 years) of the patient. On the other hand, the delay might reflect a difference in the rate of utilization of the active principles of liver extract and the animal protein factor concentrate.

Whether the second small reticulocyte response that followed the administration of the 10-unit liver extract to patient E. G. indicates that the liver extract was more effective than the animal protein factor concentrate is difficult to determine, since to permit subsidence of reticulocytosis the patient was without therapy for nine days after the course of animal protein factor concentrate was concluded. In any event, the animal protein factor concentrate, given parenterally, produced an almost theoretically maximal reticulocyte response and an increase in the levels of erythrocytes, leucocytes, platelets, and hemoglobin, in addition to causing satisfactory clinical improvement.

Conceivably, the bacterial extract contains forms or complexes of the antipernicious anemia factor that are utilized by the chick as sources of animal protein factor activity but are less effectively utilized by the human patient as sources of antipernicious anemia factor activity.

In the second patient the reticulocytes failed to attain a theoretically maximal level, but there, also, there was rapid improvement in the levels of erythrocytes, leucocytes, and hemoglobin, in addition to a satisfactory clinical response.

These findings show that a concentrate prepared from a bacterial filtrate contains a substance capable of producing an hematologic and clinical response in pernicious anemia. Whether the substance is identical with the anti-pernicious anemia factor or the recently isolated vitamin B_{12}[7-9] shown to be active in pernicious anemia cannot be decided at this time.

SUMMARY

Concentrates of microbiologically produced material, highly active as a source of the animal protein factor, as measured by assay with chicks, were shown to be effective, when given parenterally, in producing an hematopoietic response in pernicious anemia.

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EXPERIMENTAL AND CLINICAL STUDIES OF NEOHETRAMINE, A NEW ANTIHISTAMINIC AGENT

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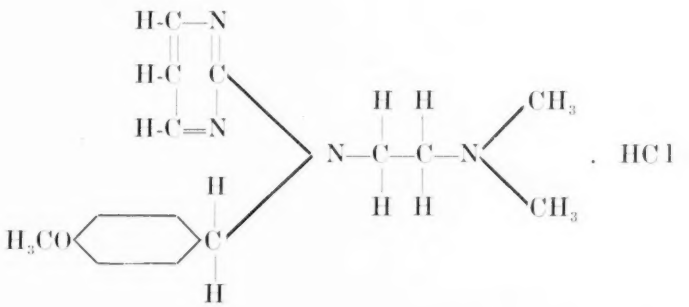
NEOHETRAMINE is one of a new group of synthetic antihistaminic agents found to produce symptomatic benefit in certain allergic states. It is the methoxy derivative of an earlier preparation, Hetramine,[1] and is the pyrimidine isostere of another potent antihistaminic drug, Neoantergan.[2]



2-(N-dimethylaminoethyl-N-p-methoxybenzyl)-aminopyrimidine monohydrochloride (Neohetramine)

The present report deals with the antihistaminic and antianaphylactic properties of Neohetramine as determined in the intact guinea pig, as well as the clinical results observed in a large group of patients with various allergic syndromes.*

EXPERIMENTAL

Antihistaminic Effect in Guinea Pigs (Table I).—Antihistaminic activity was determined by treating guinea pigs with Neohetramine fifteen to twenty minutes prior to a toxic dose of histamine; 0.4 mg. per kilogram of histamine, calculated in terms of the base, was found to be uniformly fatal when injected into the penile veins of untreated guinea pigs. All animals which received 5 mg. per kilogram of Neohetramine intraperitoneally prior to this shocking dose of histamine lived, while 50 per cent of those treated with 1.0 mg. per kilogram of the drug survived this ordinarily lethal dose of histamine.

Antianaphylactic Effect in the Guinea Pig (Table II).—Guinea pigs, 400 to 500 grams in weight, were sensitized by the subcutaneous injection of 0.1 c.c.

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*Neohetramine was supplied by the Department of Medical Research, Nepera Chemical Co., Inc., and is now distributed by Wyeth, Incorporated, Philadelphia, Pa.

TABLE I. PROTECTIVE ACTION OF NEOHETRAMINE IN HISTAMINE SHOCK

NUMBER OF GUINEA PIGS	NEOHETRAMINE (MG./KG. I. P.)	HISTAMINE (MG./KG. I. V.)	PER CENT SURVIVAL
15	None (control)	0.4	0
8	1.0	0.4	50
10	3.0	0.4	40
15	5.0	0.4	100

of horse serum and were given a challenging dose of 0.5 c.c. in a penile vein twelve days later. Protection against fatal anaphylactic shock was determined by giving Neohetramine intraperitoneally fifteen to twenty minutes prior to the shocking dose of antigen. All untreated controls died in typical anaphylactic shock. Seven out of ten animals which received 3.0 mg. per kilogram of Neohetramine lived, while only four out of ten of those pretreated with 1.0 mg. per kilogram of the drug survived. All guinea pigs receiving 0.1 mg. per kilogram of Neohetramine died.

TABLE II. PROTECTIVE ACTION OF NEOHETRAMINE IN ANAPHYLACTIC SHOCK

NUMBER OF GUINEA PIGS	NEOHETRAMINE (MG./KG. I. P.)	PER CENT SURVIVAL
14	None (control)	0
10	0.1	0
10	1.0	40
10	3.0	70

CLINICAL

Dosage and Toxicity.—Clinical experience with Neohetramine indicated that symptomatic benefit, if it occurred, would become evident within thirty minutes after ingestion of the drug and last for several hours. Patients usually were instructed to take the drug at four- to six-hour intervals, but in some instances it was found necessary to use the drug more frequently in order to control symptoms. Those with periodic difficulty were advised to take the drug only when symptoms occurred. Initially 50 mg. doses were prescribed and later were increased to 100 mg. if clinical benefit was not evident. The optimum dose for most adults was found to be 100 mg., while 50 mg. doses were employed with good results in children 6 to 12 years of age. Proportionately smaller amounts were used in younger children.

The incidence of side action from Neohetramine was less than that seen with other antihistaminic drugs previously studied.[3] Several patients in this series who previously were found unable to tolerate antihistaminic medication were able to take clinically effective doses of Neohetramine without difficulty. Among 140 patients who were given Neohetramine, only seventeen (12 per cent) complained of side effects. Drowsiness, which is the most frequently encountered untoward reaction from antihistaminic drugs, was noted by five patients; five others reported gastrointestinal irritation; three complained of vertigo; and one patient each experienced weakness, tinnitis, diplopia, and pruritis. In no instance was the side action of a severe degree. Evidence of chronic toxicity as

determined by repeated blood counts and urine examinations was not encountered in those using the drug over longer periods of time. Periodic examinations in four patients who received an average dose of 200 mg. daily for six months showed no abnormalities in urine or blood count.

Symptomatic Effect (*Table III*).—Neohetramine was used in 140 patients with one or more of the following allergic complaints: seasonal hay fever, nonseasonal allergic rhinitis, urticaria and angioneurotic edema, asthma, allergic

TABLE III. CLINICAL RESULTS WITH NEOHETRAMINE

CONDITION TREATED	NUMBER OF CASES	HELPED		NOT HELPED	
		NUMBER	PER CENT	NUMBER	PER CENT
Bronchial asthma	40	11	27.50	29	72.50
Vasomotor rhinitis	50	26	52.00	24	48.00
Hay fever	58	37	63.82	21	36.18
Urticaria					
Acute	6	6	100.0	0	0.0
Chronic	4	2	50.0	2	50.0
Dermatitis					
Atopic	3	0	0.0	3	100.0
Contact	2	1	50.0	1	50.0
Unclassified	4	3	75.0	1	25.0
Allergic headache	3	0	0.0	3	100.0
Allergic conjunctivitis	1	0	0.0	1	100.0

dermatitis, headache, and conjunctivitis. Thirty of these subjects had two allergic syndromes, such as rhinitis and asthma, or hay fever and urticaria, and since the effect of Neohetramine on each was not necessarily the same in that patient, each symptom is listed separately in the accompanying table.

Seasonal Hay Fever: A beneficial effect on rhinorrhea, itching, and sneezing was seen in 64 per cent of patients with acute hay fever due to pollen or fungus spores. In common with other antihistaminic drugs, the relief following each dose was slight in some instances and marked in others, but seldom complete. The many factors which influence symptoms in hay fever also affect the response to these drugs. Benefit is usually more evident early in the pollen season and on days when pollen or mold concentration is low. In general, patients with mild symptoms, or those with some degree of immunity through desensitization therapy, obtain more relief than those with severe symptoms.

Allergic Rhinitis, Nonseasonal: Fifty-two per cent of this group obtained some symptomatic benefit from the drug. Rhinorrhea and sneezing were usually more favorably affected than nasal blocking.

Urticaria and Angioneurotic Edema: Relatively few of these cases are included in this series. In six patients with acute urticaria, marked symptomatic action was apparent following the use of Neohetramine. Two patients with chronic urticaria were consistently relieved by the drug, while two others failed to obtain any appreciable help.

Asthma: Approximately 27 per cent of asthmatic patients reported some help from the use of the drug. The degree of benefit in these patients, in our opinion, was not striking.

Miscellaneous: The drug was occasionally helpful in alleviating the pruritis associated with allergic dermatoses. Three patients with headache and one with conjunctivitis, all of allergic etiology, were not relieved by the drug.

COMMENT

Neohetramine compares favorably with other synthetic antihistaminic drugs previously studied. While antihistaminic and antianaphylactic activities as demonstrated in the intact guinea pig are somewhat less than those determined for several other drugs of this series, its symptomatic action in allergic states is approximately equivalent to other compounds when employed in the optimum dosage of 100 milligrams.[3] A decided advantage which Neohetramine enjoys is the relative freedom from severe side effects accompanying its use. It appears to be especially indicated in those patients who are found to have a low tolerance for other antihistaminic drugs.

Experience with antihistaminic therapy has shown a rather wide individual variation in the clinical response to these compounds. A trial of several drugs in the same patient frequently will reveal one which is particularly effective and well tolerated. The incidence of symptomatic benefit from such medication is therefore increased by the availability of the newer members of this group of synthetic compounds. Our clinical experience would indicate that Neohetramine is a valuable addition to this growing list of antiallergic agents. It must, of course, be remembered that antihistaminic drugs are purely palliative medication and demonstrate no curative action in allergic disease.

SUMMARY

Neohetramine, [2-(N-dimethylaminoethyl-N-p-methoxybenzyl)-aminopyrimidine monohydrochloride], prevented fatal shock from intravenous histamine in the intact guinea pig. A similar protective action was demonstrated in guinea pig anaphylaxis.

Oral administration of the drug afforded symptomatic relief to many patients with seasonal and nonseasonal allergic rhinitis, allergic dermatoses, asthma, urticaria and angioneurotic edema.

A relatively low incidence of side effects occurred with the use of Neohetramine. It was found particularly useful in patients unable to tolerate other antihistaminic drugs.

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TRICHOSTRONGYLUS INFECTION IN HUMAN BEINGS; NEED OF DIFFERENTIATION FROM HOOKWORM

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IN 1916 Ransom,[1] in discussing the transmissibility of certain nematodes of ruminants to man, stated that Trichostrongylus was not an exotic form occurring in remotely distant regions but a parasite which had a direct and immediate interest for American physicians. Though only a few cases of Trichostrongylus infection in human beings have been reported in the American literature, we believe that this is due to a lack of knowledge regarding the differentiation of these ova from those of hookworm rather than to the rarity of the infection. In casual examinations of feces, Trichostrongylus ova can be, and probably often have been, mistakenly identified as slightly atypical hookworm ova.

Loos[2] (1895) was the first to divide Trichostrongylus into four species. Jimbo[3] (1914) undertook a statistical study of the spread of parasitic illness among the Japanese through feces examinations. He found that in Japan, where ancylostomiasis is especially prevalent, there were many people who had a peculiar type of Ancylostoma eggs which because of their great resemblance to Ancylostoma had been unjustly identified as such. The specimens appeared different from those described by Loos and therefore were named *Trichostrongylus orientalis*. Jimbo could not state what pathologic importance these worms had, but did recommend the use of oleum chenopodium as a therapeutic measure. Sandground[4] (1936), who had infected himself with Trichostronglylus larvae to determine their longevity, found that tetrachlorethylene and carbon tetrachloride were ineffective in expelling the worms. There was no diminution in the number of eggs after eight and one-half years. In 1938 Schenken and Moss[5] reported the first case of human infection with *T. colubriformis* in the Western hemisphere. They found a single adult worm in a surgically removed appendix. No definite information could be obtained regarding the source of the infection. Maplestone[6] (1941) found a 9 to 25 per cent infection rate in India, with a 10 per cent infection rate among 50 Europeans. He considered the recognition of Trichostrongylus important. Patients were often referred to as suffering from incurable hookworm infection because they were still passing eggs after several treatments with recognized efficient hookworm drugs. Because of failure to recognize the eggs, the patients had received thymol, carbon tetrachloride and oil of chenopodium, which do have a certain degree of danger. Tsuchiya and Reller[7] (1945) found Trichostrongylus ova in a patient who had worked as a farm laborer in the southern states. They though it conceivable that the infection might have occurred through accidental ingestion of larvae

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from contaminated soil. Criteria for diagnosis of the presence of Trichostrongylus ova and for differentiation from several other types of ova have been presented by them.[7] O'Neal and Magath[8] (1947) reported three cases. Two of the patients had lived in the Belgian Congo and French equatorial Africa until four months before coming to the clinic, and the third had lived in Korea for thirteen years. Williams[9] (1947) found one case (0.2 per cent) among 553 ex-prisoners returned from Hong Kong.

The following history is presented because of the rare reports of human infection, the difficulty in recognizing the ova, and the occurrence among two members in a family of five (only four of whom could be examined).

J. L., a 23-year-old white woman, was admitted to Niagara Sanatorium March 10, 1946, with a history of influenza four months before admission and a one-month history of productive cough, chest pain, night sweats, and fatigue. The admission x-ray showed an area of infiltration at the right first anterior space and gastric lavage revealed acid-fast bacilli. With bed rest the infiltration became smaller during May and June and gastric lavages were negative. The patient's temperature, however, continued to range up to 99.2°. In July it was learned from the patient's father that she had had diarrhea for the past several years. The patient, during questioning at admission, had revealed no abnormalities in regard to bowel movements. Her initial differential blood count had shown four eosinophiles, which had been accepted as a high normal. A repeat count showed seven eosinophiles. Two feces examinations were positive for ova resembling *Necator americanus*. Sputa examinations were negative for ova or parasites. The patient was then given three hourly doses of oil of chenopodium. The feces continued to show ova and the temperature remained elevated. Shortly thereafter the patient began to show blood-streaked sputum and because of increasing disease required a right pneumothorax. It was not until Oct. 14, 1946, therefore, that she was given a second course of oil of chenopodium. This course had as little effect as the first. The eosinophile count was thirteen and the temperature was usually 99°, occasionally going up to 99.6°. The laboratory, meanwhile, had continued its study of the ova because of their large size and the fact that the segmentation appeared too high for hookworm ova which usually show eight segments. These ova showed morulation up to thirty-two segments. The ova were identified as those of a Trichostrongylus species.* On Nov. 22, 1946, the patient finally consented to an examination by a proctologist. Scars due to an old ulcer as well as several small bleeding areas were found in the rectal membrane. On the basis of these findings and the past history, the patient's condition was considered to be chronic amebic dysentery. It was advised that this possibility be ruled out by the use of emetine hydrochloride. The drug, ⅓ grain, was given three times daily for five days. Feces examinations continued to show only Trichostrongylus ova and there was no change in the temperature or the eosinophile count. On Jan. 2, 1947, the patient was started on a ten-day course of carbarsone, 3½ grains twice daily. The temperature, which had been up to 99.6°, became normal on the fourth day of treatment and continued so through the course. On the first day after discontinuation of the drug the temperature again rose to 99.6° but the sedimentation rate and eosinophile count showed a slight drop. Carbarsone was started again on Jan. 28, 1947, but had to be discontinued on Feb. 6 because of nausea. This time the temperature rose from an occasional 99° to 99.4°; the sedimentation rate increased from 15 mm. per hour to 28 mm. per hour, and there was an eosinophile count of six. Trichostrongylus ova were still present and there was no evidence of *Endamoeba histolytica*. In March, 1947, proctoscopic examination of the patient was again made. There was no change from the previous examination, and Trichostrongylus was ignored as a possible cause of the findings. This time gentian violet, ½ grain three times daily, was advised. There was a slight temperature elevation and a slight increase in the number of bowel movements, as well as

*A specimen was sent to Washington University School of Medicine, St. Louis, Mo., and the diagnosis was confirmed by Dr. H. Tsuchiya.

A. *B.*

Fig. 1.—*A*, Trichostrongylus sp. ovum from daughter (Patient J. L.) (approx. ×700). *B*, Trichostrongylus sp. ovum from mother (approx. ×700). This picture reveals disintegration of the intracellular structure because it was prepared from a specimen which had been preserved for a considerable time. It is included, however, to emphasize the size which the egg may attain.

Fig. 2.—Trichostrongylus sp. larva (approx. ×700) from daughter (Patient J. L.).

abdominal cramps and nausea. Therapy was discontinued on the sixth day. Prior to this treatment the patient had noted symptomatic improvement with the use of bland diet and Amphojel. At this time she refused to try any further drugs and was continued on this regimen until discharge on May 29, 1947. At the time of discharge there were occasional loose bowel movements, rare ova of Trichostrongylus in the feces, a sedimentation rate of 30 mm. per hour, two eosinophiles in the differential count, and no evidence of anemia. The sputum had shown few to many colonies of acid-fast bacilli during the flare-up in September, 1946, but for six months prior to discharge all concentration and cultural examinations of sputa and gastric lavages had been negative for acid-fast bacilli. The pulmonary disease appeared arrested with pneumothorax.

When the ova had been properly identified, the patient's family were advised to have a checkup. Two brothers failed to show any ova on two occasions. The patient's mother, however, had many Trichostrongylus ova in December, 1946, and a few ova and an eosinophile count of seven in January, 1947. The mother reported that she had always been in good health. As far as she could remember she had never had any abnormal bowel movements. A specimen of the mother's feces was sent to the Army Medical School at Washington, D. C. The report stated that Trichostrongylus ova were present, again confirming the diagnosis.

COMMENTS

Detection of the ova of Trichostrongylus is difficult due to the small number usually present. A concentration and levitation technique may be required (Willis[10]). They are frequently mistaken by casual observers for the ova of hookworm, which they closely resemble. Trichostrongylus ova are characterized by a transparent shell membrane which is thicker and more lustrous than that of the hookworm. They are elongated, with the ends more pointed than those of the hookworm, though a great number will show some rounding at one end—like a hen's egg (Maplestone). The ova of hookworms average about 64 μ in length and 41 μ in width, while those of Trichostrongylus are much longer and slightly wider. Jimbo found the ova to average 83 to 90 μ in length and 47 μ in width; Tsuchiya and Reller: 81 to 97 by 40 to 53 μ, with an average of 86 by 43 μ; O'Neal and Magath: 76 to 86 by 44 to 47 microns. The ova of Patient J. L. in this report averaged 91 μ in length (range 80 to 100) and 44 μ in width (range 41 to 50), and those of her mother 88 μ in length (range 80 to 98) and 43 μ in width (range 40 to 46). The morula stage of Trichostrongylus will show at least sixteen divisions, a somewhat later stage of segmentation than that of hookworm ova. The individual divisions are equal in size, almost circular, and of a grapelike appearance. This is an important aid in differentiation. Two larvae were found, measuring approximately 285 μ in length and up to 24 μ in width. Jimbo has described the roundworm as being thin, delicate, sexually divided, of colorless to gray-white appearance, ranging from 380 to 670 μ in length and up to 83 μ at its widest portion.

According to Craig and Faust[11] several hundred worms are necessary to provoke marked clinical manifestations. Jimbo also notes that the severity of symptoms will depend on the number of parasites. Maplestone, however, states that as far as he knows the worms never give rise to any objectionable symptoms. Two of O'Neal and Magath's patients had intermittent blood-tinged stools, while the third had abdominal cramps and frequent loose stools. The daughter (Patient J. L.) in our cases had diarrhea for several years while the mother had no symptoms. Yet the mother on occasions showed showers of ova.

Since so few cases of human Trichostrongylus infection have been reported, the mode of infection is not definitely known. Chandler[12] believes that the infection occurs through the ingestion of contaminated vegetable matter rather than by penetration through the skin. It is possible that grazing lands are being seeded with eggs, since goats and sheep are natural hosts. Koino,[13] in experiments on mice, traced the migration of the larvae and they seemed to follow the same route as hookworm larvae. He obtained penetration and migration to the lungs both by the oral route and through the skin. It would seem probable that human beings could acquire Trichostrongylus infection in the same manner as they do hookworm.

From all reports, it appears impossible to eradicate the Trichostrongylus with the usual anthelmintics. As seen from previous investigations and from our own efforts, the use of oil of chenopodium, thymol, carbon tetrachloride, tetrachlorethylene, as well as emetine hydrochloride, carbarsone, and gentian violet is ineffective. Where Trichostrongylus has been mistaken for hookworm, the patients have been subjected to needless, objectionable, and perhaps dangerous treatments.

CONCLUSIONS

There are, we realize, a number of questions left unanswered in this report. We do not know how the infection was acquired. We do not know whether the infection was acquired simultaneously by mother and daughter, or whether it was transmitted from one to the other.

We cannot offer any course of therapy for the eradication of Trichostrongylus. The usual anthelmintics have been found ineffectual in the so-called incurable hookworm infection, which may be a Trichostrongylus infection.

In areas endemic for hookworm, as well as in individual cases, the ova of hookworm should be carefully examined and differentiated from those of Trichostrongylus. A lack of knowledge regarding the latter led to an original diagnosis of *Necator americanus* in our patient. If remembered in differential diagnosis, however, the shape, larger size, and advanced segmentation of the Trichostrongylus ova cannot fail to attract the attention of the investigator.

The significance of the infection as related to the future health of patients can only be determined by prolonged follow-up and by further case reports.

We wish to thank Dr. H. Tsuchiya, Washington University School of Medicine, St. Louis, Mo., and Lt. Col. G. W. Hunter and Major R. Traub of the Army Medical School, Washington, D. C., for checking the identity of the ova, and Mr. M. Diedric, the University of Buffalo Medical School, Buffalo, N. Y., for taking the photographs.

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LABORATORY METHODS

ERYTHROCYTES IN URINARY SEDIMENT: IDENTIFICATION AND NORMAL LIMITS

With a Note on the Nature of Granular Casts

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ALTHOUGH the importance of erythrocytes in the urine has long been recognized and their significance a subject of much discussion, there has been little attempt to confirm their identification. The average laboratory technician fails to note the presence of red blood cells unless their number is definitely above the upper limits of normal.[1] Unfamiliarity with the varied forms of erythrocytes in urinary sediments, cursory examinations, and inadequate technique are the main reasons for this failure. Addis[2] noted that care was necessary to avoid passing over fragmented or partially lysed cells which may cast only faint shadows. The importance of the problem is emphasized by a 2 per cent incidence of hematuria in 20,000 consecutive patients in clinic practice[3] and a 10 per cent occurrence of erythrocytes in urinary sediments examined in our laboratory.

Two approaches have been used in this country to aid detection and to cope with the problem of identification: (1) the quantitative work of Addis, and (2) tests for hemoglobin and related substances, utilizing benzidine or orthotolidine. The first technique is tedious and inconvenient for clinical use, while in the second the reagents may react with interfering substances and do not morphologically identify erythrocytes. Aware of the confusing variations in size, shape, and optical density of erythrocytes in the urinary sediment and of the responsibility of even an experienced technician for correct identification, we have modified a tissue stain[4] which appears to be specific for red blood cells.

Stock solutions:

1.0 per cent aqueous solution of benzidine hydrochloride (Merck).
2.0 per cent aqueous solution of sodium nitroprusside.
30.0 per cent hydrogen peroxide (superoxol).
The benzidine and nitroprusside solutions keep from four to five months in brown bottles, if not exposed to direct sunlight.

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Working solutions:

A. 0.1 per cent benzidine in 0.2 per cent nitroprusside.
1 c.c. of the 1.0 per cent aqueous solution of benzidine (stock) is diluted to 6 to 7 c.c. with distilled water.
1 c.c. of the 2.0 per cent aqueous solution of sodium nitroprusside (stock) is added and then distilled water to 10 c.c.
This solution is stable for about two weeks; it should be discarded as soon as a precipitate is formed.

B. 3.0 per cent hydrogen peroxide.
1 c.c. of superoxol is made up to 10 c.c. with distilled water.
This solution deteriorates in from three to four days.

TECHNIQUE

A drop of sediment is placed on a slide and a drop of benzidine-nitroprusside solution is added. Thorough mixing is accomplished by tilting the slide or by stirring with the cover glass. A drop of dilute peroxide solution is then added and carefully mixed.

Sediments of alkaline specimens, which do not stain readily, may be treated within the tip of the centrifuge tube with 1 or 2 drops of 10 per cent nitric acid to destroy the crystals and lower the pH. In our experience this has not caused hemolysis of red cells, probably because of the fixing property of urine and the presence of basic salts. After three washings with 0.9 per cent saline solution these sediments give results comparable to those attained in acid urines. Urine specimens of a high specific gravity, especially those containing preservatives, may react slowly to the stain, and should stand five to eight minutes before examination. Washing three times with 0.9 per cent saline solution should accelerate staining.

RESULTS

The erythrocytes are stained a deep blue-purple. This color may be pale, even a light blue if the cell has deteriorated. Some cells will have scattered deep blue punctate granules. Suspensions of yeast cells in normal urine less than twenty-four hours old may show a slight blue-green iridescence which is not confused with the deeper color and less refractive appearance of the stained erythrocytes, while those over twenty-four hours old have no affinity for the stain. Leucocytes may take a pale-gray appearance in the cytoplasm with slightly dark and irregular staining of the nucleus. No granules have been observed in the cytoplasm of white blood cells or epithelial cells. Hyaline casts do not take the stain. The granules of fine and coarse granular casts have the deep bluish-purple hue of stained erythrocytes. The red blood cells of casts stain similarly to those free in solution. Fungi and spores show no response to the stain.* Pollen, diatoms, prostatic bodies, and other elements of urine have no affinity for the stain.

*We are indebted to Mr. Robert K. Steffa, Waterloo, Ia., for submitting fifteen species of fungi and three strains of yeast.

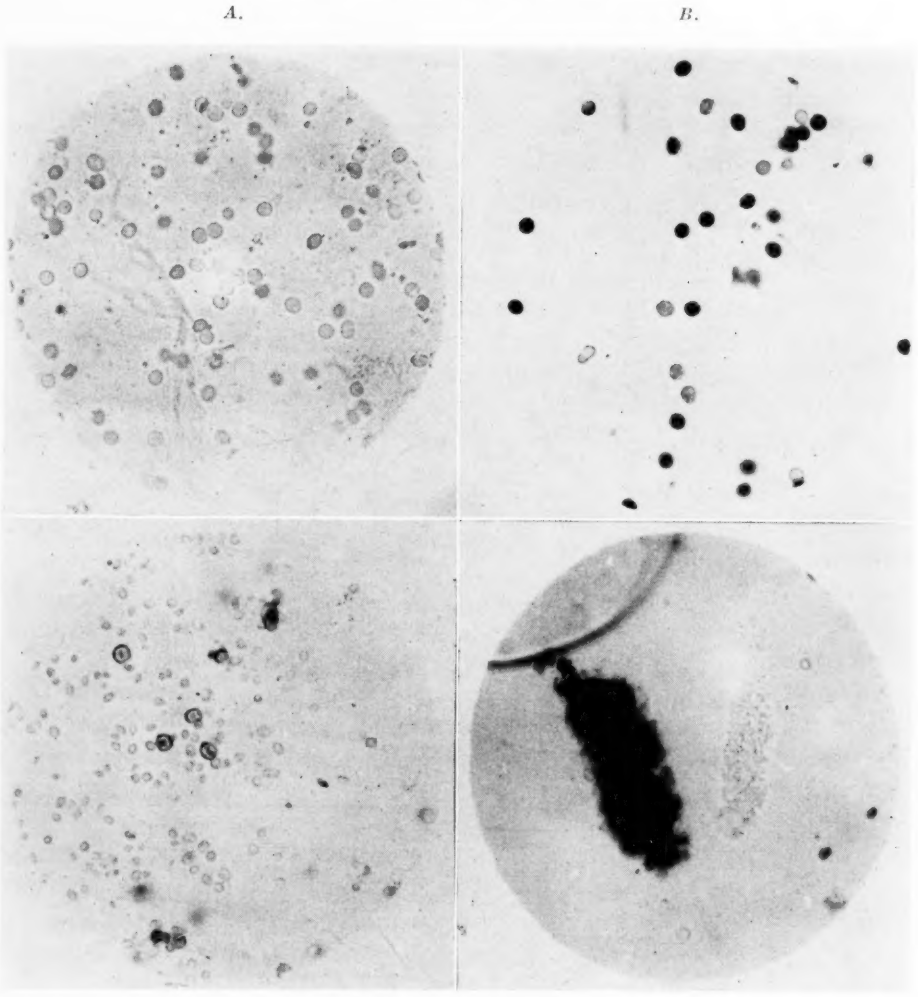

Fig. 1.—*A*, Red cells, unstained. *B*, Same cells, stained. Note variable degree of stain intensity. *C*, Suspension of yeast and red cells, stained. *D*, Composite print. Granular casts (and few red cells), stained and unstained.

STAINING REACTION

Since this stain seems to have a specificity for red blood cells, the probable site of action is hemoglobin or one of its heme derivatives. Benzidine and oxidizing agents, such as hydrogen peroxide, react with acid solutions of hemoglobin to produce a blue color which, according to Wu,[5] is exactly proportional to the amount of hemoglobin present. Bing and Baker[6] consider this a pseudoperoxidase reaction. Benzidine and hydrogen peroxide react slowly with intact red cells in the urine to produce after some time a faint, blue color. Sodium nitroprusside greatly increases the speed and intensifies the color of the reaction.

The mechanism of this reaction is very complex. It is known, however, that solutions of benzidine subjected to nascent oxygen or sodium ferricyanide

rapidly form a deep blue precipitate. Solutions of benzidine and sodium nitroprusside form a similar precipitate only after standing for days. Benzidine, a fundamental dyestuff, is attached to the protein of red cells. Hemoglobin containing ferrous iron (Fe^{++}) is oxidized to methemoglobin containing ferric iron (Fe^{+++}) by hydrogen peroxide.[7] The nitroprusside might react by forming a colored iron-cyanide compound or alter the reaction by its nitro group. Since we obtained no results under the conditions of our stain substituting sodium ferricyanide, sodium ferrocyanide, or nitrites, it appears that nitroprusside may be a catalyst.

Because of these findings we feel that benzidine may be an indicator of an oxidation-reduction system[8] which results in the oxidation of benzidine and the development of a deep blue color. The nitroprusside may act as a catalyst.

DISCUSSION

Identification.—Most efforts at identifying blood in the urine have been directed toward the development of color tests for hemoglobin and related substances. Benzidine,[9, 10] tolidine,[11] and orthotolidine[1, 12] have been used as the reagents. These substances vary in their reactions with the pH of the urine,[13] ascorbic acid level,[12, 14] presence of iodides and bromides,[12] yeast or pus cells.[10] Some investigators[1, 11, 12] have correlated the color reactions with quantitative erythrocyte counts of urinary sediments and claim that positive results occur only in the presence of a significant number of red blood cells. Others fail to find this correlation.[15] Endtz[16] has identified erythrocytes in the urinary sediment utilizing a benzidine stain. We found this technique to be slower acting and to give less intense color than the benzidine-nitroprusside method. The common practice of producing hemolysis of red blood cells by the addition of acetic acid to the sediment is unreliable, particularly in specimens containing preservatives.

To corroborate the varied appearance of red cells observed in submitted specimens, samples of normal blood were suspended in varying concentrations of sodium chloride with specific gravities of 1.004 to 1.040 with and without preservative tablets.* Studies of the sediments from these solutions substantiate our opinion of a great variation in size and appearance of red blood cells in urine specimens of different specific gravities and salt concentrations. The sediments from suspensions containing the preservative tablet had a more constant form of erythrocyte than those without the preservative tablet. Although the tablet liberates formaldehyde in solution, no inhibiting reaction upon the stain was observed. The buffer and slight acidifying effect of the preservative tablet creates a condition favorable to staining.

Of particular interest was the finding that the granules of fine and coarse granular casts stain the same dark bluish-purple of red cells and red cell debris which was found in the sediment of red cell suspensions. This would tend to

*Preservative tablet No. 4, Metropolitan Life Insurance Company specifications, containing potassium acid phosphate, sodium benzoate, benzoic acid, Urotropin, sodium bicarbonate, mercuric oxide red.

indicate that the granules are particles of red blood cells which have been destroyed in the kidney and incorporated in the substance of the cast. This helps to explain the more serious implication of granular casts.

The efficacy of various preservative materials was evaluated by preparing suspensions of red cells in urine with preservative tablet, thymol, toluene, chloroform, boric acid, and formaldehyde. None of these materials inhibited the staining reaction, but only the formaldehyde and the preservative tablet satisfactorily preserved erythrocytes. From our suspension of erythrocytes in urine with one preservative tablet per ounce we obtained 100 per cent recovery of red blood cells even after seventy-two hours. This is greater than the 50 to 80 per cent recovery estimated by some investigators.[1] We have obtained identical counts per high-power field and have had positive staining reactions on specimens that have stood at room temperature for as long as four to five months. There must be some breakdown of red cells in the kidney, however, as our findings on the nature of the granules of granular casts suggest.

Normal Limits.—We were further interested in determining the number of red blood cells in the urinary sediment that may be considered within normal limits. Since the majority of urinalyses are on single voided specimens, we endeavored to correlate the results of quantitative studies with those that might be expected on sediments from random specimens. It would appear that a normal individual may excrete as many as 600,000 red blood cells per twelve-hour period.[2, 15, 17] This number of erythrocytes suspended in 300 c.c. of urine, the average twelve-hour output of the quantitative studies, gave about 2 red blood cells per high-power field by our standard technique. This technique consists of centrifugation of 15 c.c. of urine at 2,000 revolutions per minute for five minutes and examination of twenty high-power fields of a drop of sediment placed beneath a ⅞ inch cover slip. Although the findings of single voided specimens may vary,[18] it would appear that the repeated presence of more than 2 red blood cells per high-power field in the centrifuged sediment may indicate an increased loss of erythrocytes from one of the many sources of occult bleeding in the urinary tract. This estimate is supported by the laboratory findings in 3,000 consecutive single voided specimens from young men applying for employment. In this series 2,484 specimens were negative for erythrocytes. In sixty instances, or only 2.0 per cent, the specimens contained 2 to 3 red blood cells per high-power field, and only twenty-three samples, or .7 per cent, contained 4 to 5 red blood cells per high-power field. These values support our findings on the correlation of quantitative studies that the repeated excretion of more than 2 red blood cells per high-power field may be significant.

The count per high-power field obtained by adding the staining solutions to the sediment directly upon a slide is lower than that of an untreated sediment. To avoid dilution, the reagents may be added to the sediment within the tip of the centrifuge tube, carefully mixed, recentrifuged, and the supernatant fluid decanted. The sediment thus prepared will have approximately the original dispersion of red blood cells. In performing quantitative counts, the staining solution may be used in diluting to the desired volume in the Addis centrifuge tube.

SUMMARY

A benzidine-nitroprusside stain for erythrocytes in the urinary sediment is described which we believe to be specific for red blood cells and fragments thereof. With this technique the red blood cells stain a deep blue-purple and are easily distinguished from contaminants and other formed elements of urine.

The stain is based upon the pseudoperoxidase reaction of hemoglobin and benzidine. The nitroprusside may act as a catalyst.

The granules of fine and coarse granular casts take on the same bluish-purple stain. This finding suggests fragments of red blood cells as the origin of the granules and helps explain the more serious implication of granular casts.

Erythrocytes may occur in the urinary sediment of normal individuals up to approximately 2 per high-power field. This level, determined by a conversion of the results of quantitative studies to the methods employed in analyzing single specimens, is supported by the results of our findings in 3,000 consecutive urinalyses in young men applying for employment. In this series, only 1.2 per cent of specimens contained more than 2 red blood cells per high-power field.

The preservative tablet and formaldehyde are efficient agents for the preservation of erythrocytes. Thymol, toluene, chloroform, and boric acid are not satisfactory. None of these substances interfere with the stain for erythrocytes.

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THE SPECTROPHOTOMETRIC DETERMINATION OF BLOOD pH

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PROBABLY the most frequently used methods for the determination of blood pH are those of Hastings and Sendroy,[1] Hawkins,[2] and Shock and Hastings.[3] In recent years the electrometric determination of pH has been brought to a high degree of accuracy under certain well-controlled conditions, especially by Nims.[4] However, for purposes of clinical research the preceding visual colorimetric methods usually are considered adequate. There is one drawback which detracts from their usefulness—the observer must be experienced in the visual matching of colors. While this usually is not difficult with normal clear serum or plasma, slight amounts of hemolysis, lipemia, or bilirubinemia often confuse the inexperienced observer. In addition, there are certain individuals who have great difficulty in distinguishing the difference between density and hue of color.

It was with these difficulties in mind that an objective spectrophotometric method of determining blood pH was sought for. Evelyn[5] has described such a method, but there is no technical provision for comparison with other methods. Therefore it was decided to apply spectrophotometric measurements to the method of Hastings and Sendroy[1] in such a manner that comparison with the visual method could be made on the same sample.

The principle of this method depends on the fact that the color of the indicator, phenol red, is different in acid and alkaline solutions. At a given pH the relative amounts of these two colors determine the final color of the solution. These two colors absorb light of widely different wave lengths and can be measured independently in the same solution with a suitable spectrophotometer. Since the relative amounts of these two colors are dependent upon pH, the measurement of these amounts constitutes an indirect measurement of pH.

Reagents.—The reagents are those of Hastings and Sendroy. Detailed directions for their preparation are given elsewhere.*

Procedure.—Four milliliters of the adjusted dye-saline mixture are placed in a colorimeter tube and covered with a small amount of paraffin oil. Two-tenths milliliter of serum or 0.4 ml. of blood are then added and the mixture is stirred gently with a glass rod. In the case of blood, the tube is centrifuged to throw down the cells. The blank is prepared in a similar fashion, substituting saline for dye-saline. The optical density of the sample is determined at wave lengths 565 and 420 mμ, after which the temperature of the solution is recorded.

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*Peters and Van Slyke: Quantitative Clinical Chemistry, vol. II, p. 796, 1932.

Calculation.—The ratio of optical density at 565 mμ to optical density at 420 mμ has been determined for a number of solutions of known pH. (Fig. 2.) For easy reference it is convenient to prepare such a graph from the data supplied by Evelyn.[5] After having calculated the ratio, read the pH from the graph. This value is the uncorrected pH. The true pH is obtained by applying the temperature correction[6]:

$$\text{True pH} = \text{Uncorrected pH} - (0.42 - .01\ T^{\circ}\ C)$$

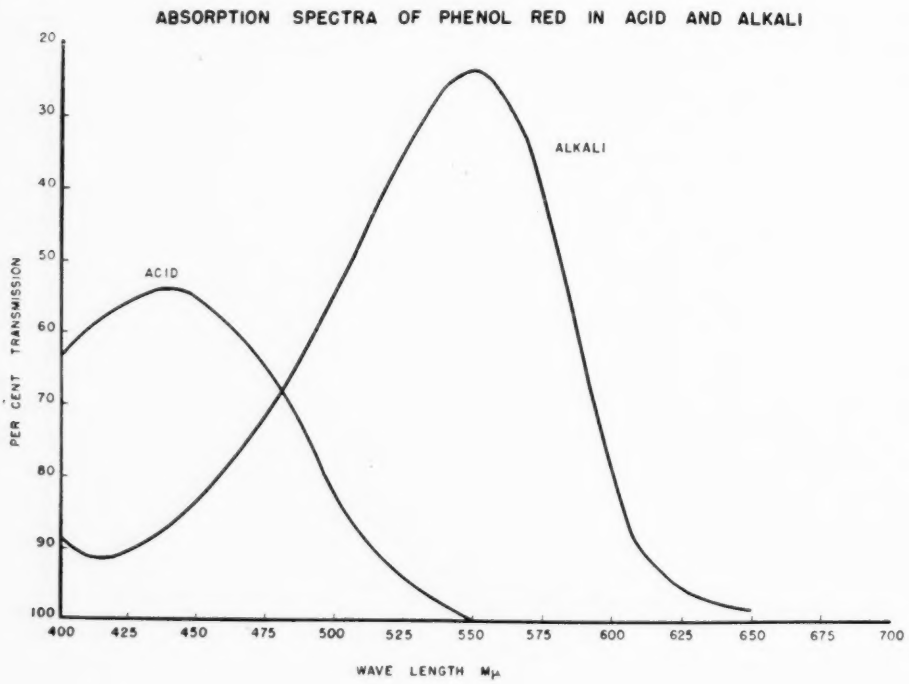

Fig. 1.

Experimental.—The absorption spectrum of phenol red in both acid and alkaline solution was determined with the Beckman spectrophotometer. To a four milliliter portion of dye-saline solution were added 0.2 ml. portions of strong acid or alkali. The transmission of the resulting solutions was determined at various wave lengths (Fig. 1). It is seen that measurements of the two colors are best taken at wave lengths 565 and 420 mμ to avoid undue interference.

In order to check the data supplied by Evelyn for the relation of pH to the ratio of optical densities in dilute solutions of phenol red, a series of Sorensen's M/15 phosphate buffers was prepared,* the pH being accurately adjusted electrometrically. To four milliliter portions of the adjusted dye-saline solution were added 0.2 ml. portions of the buffer. The optical densities of the resulting solutions were determined at wave lengths 565 and 420 mμ at 38° C.

*By Mr. J. Earle Adler.

The ratio of the densities was plotted against pH, resulting in the curve shown in Fig. 2. As was expected, when log ratio was plotted against pH a straight line resulted. These data are in good agreement with the data of Evelyn.

To compare the visual with the spectrophotometric method, the pH standards described by Hastings and Sendroy were prepared. To insure stability these standards were prepared in sodium borate and boric acid[7] instead of hydrochloric acid and sodium hydroxide. Test tubes of the same size were selected for use in the Coleman Junior spectrophotometer. The comparison was made as follows: To four milliliters of adjusted dye-saline solution in a colorimeter tube were added 0.2 ml. of serum. The usual blank was prepared and the tubes were warmed to 39° C., after which comparison was made with the visual standards.

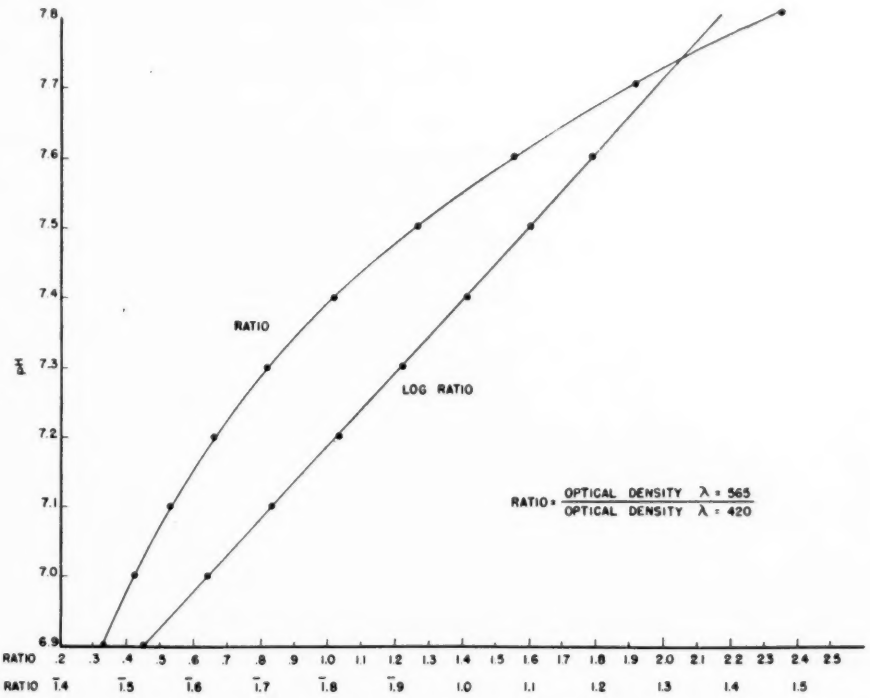

Fig. 2.—The relation between pH and the ratio of the optical densities at wave lengths 565 and 420 millimicrons.

The tubes were then allowed to cool to room temperature and were placed in the spectrophotometer where the optical densities at 565 and 420 mμ were determined. The ratio of optical densities was calculated, the corresponding pH read off the graph, and the temperature correction applied. The sera used were taken from both normal and pathologic subjects. The comparison of the two methods is seen in Table I along with other pertinent data. In some cases two or more observers participated in the visual comparison. These data indicate that the spectrophotometric method is at least as acceptable as the visual. In addition there is the added advantage that the personal error inherent in the

TABLE I. COMPARISON OF VISUAL AND SPECTROPHOTOMETRIC DETERMINATION OF pH

SUBJECT	OPTICAL DENSITY $\lambda = 565$ mμ	OPTICAL DENSITY $\lambda = 420$ mμ	RATIO	UNCORRECTED pH	T° C.	CORRECTED pH	VISUAL pH OBSERVER 1	VISUAL pH OBSERVER 2	VISUAL pH OBSERVER 3	DIFFERENCE	SERUM CO_2 CONTENT (VOL. %)
1 (Schizophrenia)	.377	.2291	1.65	7.63	32.0	7.53	7.50	7.50		.03	63
2 (Betahydroxybutyric acid tolerance test)	.374	.2200	1.70	7.64	29.5	7.53	7.50			.03	58
3 (Betahydroxybutyric acid tolerance test)	.369	.2200	1.66	7.63	33.0	7.54	7.53			.01	57
4 (Normal)	.392	.2384	1.64	7.62	25.5	7.46	7.45	7.54	7.54	.01*	51
5 (Diabetic acidosis)	.2310	.2840	0.81	7.30	25.0	7.13	7.15	7.15		.02	25
6 (Pancreatic fibrosis)	.352	.2007	1.57	7.65	33.0	7.57	7.59			.02	
7 (Pancreatic fibrosis)	.342	.2384	1.43	7.56	33.0	7.47	7.45			.02	53
8 (Diabetic acidosis)	.2403	.2798	0.86	7.33	28.0	7.19	7.20			.01	20
9 (Diabetic acidosis)	.1956	.2915	0.67	7.21	30.0	7.09	7.10	7.10		.01	18
10 (Pancreatic fibrosis)	.323	.1973	1.64	7.62	34.0	7.54	7.55			.01	113
11 (Pyloric stenosis)	.406	.1756	2.31	7.79	33.5	7.71	7.73	7.71	7.71	.00	63
12 (Pancreatic fibrosis)	.347	.1922	1.80	7.67	32.0	7.57	7.53	7.53		.04	
13 (Diabetic acidosis)	.297	.2557	1.16	7.46	30.0	7.34	7.35			.01	27
14 (Normal)	.403	.2997	1.61	7.60	27.0	7.45	7.45			.00	
15 (Normal)	.382	.2291	1.66	7.63	27.0	7.48	7.50			.02	
16 (Normal)	.301	.2596	1.16	7.46	32.0	7.46	7.45			.00	
17 (Normal)	.344	.2676	1.28	7.51	32.0	7.41	7.40			.00	
18 (Normal)	.374	.2882	1.29	7.51	30.0	7.39	7.40			.01	
									Average difference	.014	

*Because of the normal CO_2 content it would seem that the value 7.45 is the more nearly correct one.

visual method is eliminated, and this at the slight expense of the preparation of a graph and the selection of several colorimeter tubes. However, the errors of the colorimetric method per se are retained.

SUMMARY

An objective spectrophotometric method for the determination of blood pH is described. A comparison of the visual and spectrophotometric methods is presented showing an average difference of between .01 and .02 pH units.

Grateful acknowledgement is made to Dr. Alexis F. Hartmann for advice and criticism during the course of this work and in the preparation of this paper.

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A MICROMETHOD FOR THE DETERMINATION OF SERUM STREPTOMYCIN LEVELS*

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A MICROMETHOD for the assay of streptomycin in whole blood has been described by Forgacs and Kucera[1] using agar plates seeded with a variant strain of *Bacillus subtilis*, Cohn *emend* Prasmowski; blood levels are estimated from the zones of inhibition produced by .01 ml. aliquots of oxalated blood. Although it is a microtechnique for each determination, the construction of standard curves requires larger amounts of blood. Individual blood idiosyncrasies require that a separate standard curve be constructed for each patient. Furthermore, accuracy in the method requires maintenance of a uniform distribution of cells during the entire procedure, which is very difficult in bloods with low hematocrit values and elevated sedimentation rates. Since streptomycin does not enter the cells[2] and since the just mentioned difficulties seemed to stem from the presence of cells, it seemed desirable to modify the method for use with serum alone. Because of the decreased variables it was thought that one master standard curve might be generally applicable. The following technique was therefore developed.

Preparation of the Standard Curve.—The serum from 12 ml. of blood was required to determine the thirteen points on the curve. Final concentrations of 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 25, and 35 μg of streptomycin were prepared as described by Forgacs and Kucera,[1] with the exception that serum was substituted for oxalated blood. Agar plates were seeded with *B. subtilis* as in the original method.[1] The serum-streptomycin mixture was drawn up into a 0.1 ml. serologic pipette with the aid of a Guthrie pipette controller.† The meniscus was lowered to one of the graduations, and the tip was wiped dry. By careful turning of the pipette controller screw, .01 ml. was forced onto the pipette tip and touched to the agar plate which was resting on a level surface. This was repeated on each of five plates. There is space on each plate for four zones, so that each set of five plates was used for four different concentrations. The plates were incubated overnight at 30° C. The diameters of the zones of inhibition were then read with a millimeter rule. The mean of the five readings for each concentration was recorded. This procedure was carried out on thirty individual sera. The mean value for each point was obtained and plotted against micrograms of streptomycin, as shown in Fig. 1.

The statistical data are listed in Table I. At only two points, namely 5 and 15 μg per milliliter, did the number of observations falling outside the

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†Obtained from Eimer and Amend, New York, N. Y.

range of mean ± 2 σ exceed 5 per cent. Sera from all age groups (newborn to adult) were used, and no significant difference between sera was found. Thus a master standard curve such as this can be used for all determinations.

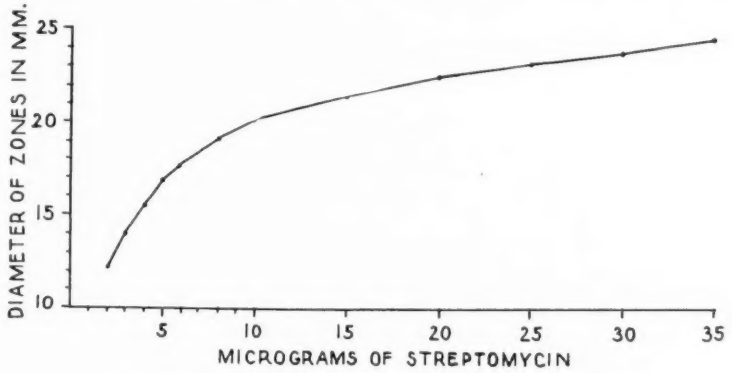

Fig. 1.—Standard curve, computed from thirty sera.

TABLE I. STATISTICAL DATA—SERUM STANDARD CURVE

STREPTOMYCIN (MG. PER ML.)	NUMBER OF OBSERVATIONS	RANGE (DIAMETER OF ZONE IN MM.)	MEAN (DIAMETER OF ZONE IN MM.)	STANDARD DEVIATION	NUMBER OF OBSERVATIONS OUTSIDE RANGE OF MEAN ±2 STANDARD DEVIATIONS
2	27	11.4—13.2	12.1	.39	1
3	28	13.2—14.8	14.0	.44	0
4	30	14.4—16.6	15.6	.52	1
5	30	15.8—17.4	16.8	.38	3
6	30	17.0—18.6	17.7	.46	0
7	30	17.8—19.0	18.4	.36	0
8	28	18.6—19.8	19.1	.40	0
10	29	19.4—20.8	20.0	.39	1
15	30	20.2—22.4	21.3	.50	2
20	29	21.4—23.0	22.3	.47	0
25	30	22.2—23.8	23.0	.43	0
30	27	23.0—24.4	23.7	.42	0
35	21	23.8—25.2	24.5	.38	0

Determination of Serum Streptomycin Level.—Capillary blood is collected. The collecting tube is made either from a three-inch length of glass tubing, with one end sealed, or from a 7.5 by 95 mm. shell vial. The open end is drawn out to a capillary tip and sealed. The wide portion of the tube is held over the flame and an air vent is quickly produced in the softened glass by the expanding air within the sealed tube. For the collection of blood, the capillary tip is broken off near its base. The finger or heel is punctured so that a free flow of blood is obtained. The tube is held in as nearly vertical a position as possible and the blood readily flows into the tube and down to the base. For each determination 0.3 ml., or a 1.5 cm. column of blood, is adequate. The tubes are stood upright until the blood is clotted; then the tube is filed off just above the surface of the blood. The clot is broken up with an applicator stick and the tube is centrifuged at 1,500 to 2,000 revolutions per minute for fifteen minutes. As

described, 0.5 ml. serum is then drawn up into a 0.1 ml. pipette and a drop of .01 ml. is applied to each of five prepared plates. The plates are incubated overnight at 30° C. The mean of the five zones is converted to micrograms of streptomycin by means of the standard curve.

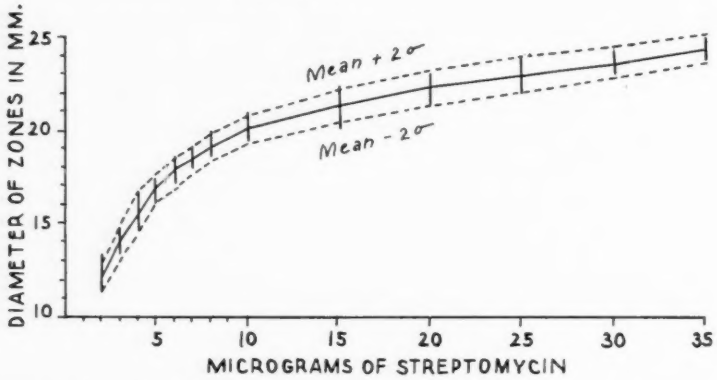

Fig. 2.—Chart showing expected variations in streptomycin values determined from given zone diameters. Solid curve represents the standard curve, as in Fig. 1. Dotted curves represent value of the mean +2 standard deviations and the mean —2 standard deviations. Vertical lines represent the range of zone diameter means obtained at each serum concentration of streptomycin. There are very few determinations falling outside the range of ±2 standard deviations.

Fig. 2 is a graphic representation of the range of error to be anticipated in the method. Rarely does the spread of observations exceed the range of 2 standard deviations. It can be seen that the least variation in estimated streptomycin level for a given zone diameter is to be found in that portion of the curve from 2 to 6 μg of streptomycin, where for clinical purposes the least deviation is desirable.

For good results, the following precautions should be observed:

(1) Media must be prepared with meticulous constancy, for slight variations in ingredients may markedly affect the activity of streptomycin.

(2) The pH of the medium must be adjusted to 7.2 shortly before the plates are poured.

(3) Results are best when flat-bottomed Petri dishes are used.* It is important that the 10 ml. of medium be measured carefully when plates are poured.

(4) Since dust particles distort the drops of serum, the room should be dust-free during the entire procedure.

(5) The spore suspension is remarkably stable and is usually reliable for several months. However, it may become contaminated, with a resulting change in sensitivity. If this method is being used routinely over a long period of time, it is well to run a weekly or fortnightly standard curve to insure reproducibility.

Even with the increased variation at higher levels, the method is sufficiently exact for clinical purposes. It must be pointed out, however, that without modifications the method is not reliable for use in a patient receiving both penicillin and streptomycin, since the strain of *B. subtilis* used is moderately sensitive to penicillin.

*Pressed glass Petri dishes were obtained from Corning Glass Company, Corning, N. Y.

SUMMARY

A method is described for estimating streptomycin in serum. It uses only 0.3 ml. of blood, and therefore is particularly applicable for use among infants and children. It requires no unusual or expensive equipment, and the laboratory techniques are easily acquired and carried out.

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A METHOD FOR THE QUANTITATIVE DETERMINATION OF BILIRUBIN IN URINE

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SEVERAL procedures for the quantitative determination of bilirubin in urine have been described. Most of these have utilized the principle of adsorption of bilirubin from urine on insoluble barium or calcium salts. Bilirubin is then eluted from the adsorbate and some characteristic color reaction is used to determine the concentration. Methods employing these principles have been described by Hunter,[1] Godfried,[2] and With[3] among others.

Our efforts to use such techniques for the quantitative determination of bilirubin proved disappointing for several reasons.

First, we were unable to effect complete removal of bilirubin from icteric urine. Pure bilirubin added to normal urine could be removed completely from solution by addition of barium chloride and centrifugation of the adsorbate, but quantitative removal of naturally occurring bilirubin from icteric urine could not be achieved by the same technique.

Second, elution of bilirubin from the barium sulfate-bilirubin complex was incomplete after one extraction.

Finally, the most disturbing problem arose in connection with the color development of the eluted bilirubin. The previously mentioned authors employed some modification of the Ehrlich diazo reaction. This reaction gives a characteristic red color with bilirubin. On diazotization, many urines develop a reddish-brown color which masks the color of diazotized bilirubin. With[3] tried to overcome this difficulty by extraction of the diazotized bilirubin with chloroform, but he reported that he was unsuccessful in obtaining quantitative extraction.

Some investigators have tried to estimate bilirubin in urine by direct diazotization without preliminary adsorption. Goodson and Sheard[4] have published such a procedure. In their procedure no means of obviating the interference due to the diazotizable nonbilirubin chromogenic material in urine is offered.

Our attempts to determine bilirubin in urine by direct diazotization in simple alcoholic solution gave promising results when highly icteric urine was tested. However, normal dark urines free from bilirubin developed a red-brown color upon diazotization. This red-brown color absorbed a significant amount of light when read in a photometer at a wave length suitable for diazotized bilirubin. A direct diazotization method, therefore, seemed to require some means of avoiding the interference of this diazotizable nonbilirubin chromogenic material.

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It seemed feasible to avoid this interference by application of the Vierordt principle[5] for determining individual concentrations of pigments in a mixture. This requires that the mixture be read at two wave lengths, that the ratios of the optical densities at both wave lengths for each pigment in pure solution be known, and that these ratios be constant over the range of concentrations to be tested. Gibson and Evelyn[6] have applied this principle to the photometric determination of Evans blue in serum in the presence of hemoglobin. Engstrom and Mason[7] and others have employed it for the photometric determination of 17-ketosteroids in urine.

Employing this principle we have developed a procedure for the determination of bilirubin in urine which overcomes interference from nonbilirubin material.

METHOD

Reagents.—

(1) Alcohol, 95 per cent ethanol, U.S.P.

(2) Diazo reagent A: Dissolve 1 Gm. sulfanilic acid in 15 ml. concentrated hydrochloric acid in a liter volumetric flask and dilute to mark with water. (Keeps indefinitely.)

Diazo reagent B: Dissolve 0.5 Gm. sodium nitrite, chemically pure, in water and make to 100 milliliters. Store solution in refrigerator and discard when it develops discernible color.

Diazo reagent for use: Mix fresh prior to determination 10 ml. diazo reagent A and 0.3 ml. diazo reagent B.

Procedure.—One milliliter of urine (as voided or diluted*) is measured into a colorimeter tube (19 by 150 mm.). Eight milliliters of alcohol and 1 ml. of freshly prepared diazo reagent are successively added. Mix. Allow thirty minutes for color development; then add 0.25 ml. concentrated hydrochloric acid and mix. Read per cent transmission in photometer† at wave lengths of 575 and 450 mμ with photometer adjusted to read 100 per cent transmission with alcohol blank at each respective wave length.

Convert the transmission readings at each wave length to corresponding optical densities.

Optical density equals 2 minus log T, where T equals per cent transmission. The optical density of bilirubin is then calculated from the equation: $Y_{575} = 1.05M_{575} - 0.202\ M_{450}$, where Y_{575} equals optical density of bilirubin at 575 mμ, M_{575} equals observed optical density of diazotized urine at 575 mμ, M_{450} equals observed optical density of diazotized urine at 450 mμ, and where the numerical constants have been derived experimentally as given elsewhere in the text.

Given Y_{575}, the concentration of bilirubin equals K times Y_{575} where K is the calibration factor obtained as described below.

Standardization With Pure Bilirubin.—

Stock standard (10 mg. per cent): Dissolve 10 mg. of pure bilirubin‡ in chloroform and make to a volume of 100 milliliters.

*In clinical testing the urine is diluted to 100 ml. per hour if the urine flow is not already greater than that.

†Photometer readings are made on the Coleman Jr. Clinical Spectrophotometer, model 6A.

‡Eastman Kodak Bilirubin No. 2101.

Dilute standard (1 mg. per cent): Dilute 10 ml. of the stock standard to 100 ml. with alcohol. Measure 0.5, 1, 2, 3, 4, and 5 ml. of the 1 mg. per cent standard into colorimeter tubes. These tubes correspond respectively to 0.5, 1, 2, 3, 4, and 5 mg. per cent. Make each to a volume of 8 ml. with alcohol. Add 1 ml. of water to each and mix. To each add 1 ml. diazo reagent; mix; allow color to develop thirty minutes; then add 0.25 ml. concentrated hydrochloric acid. Mix and read at 575 mμ against alcohol blank set at 100 per cent transmission. A transmission-concentration curve may be plotted on semilog paper or a conversion factor may be calculated from the corresponding optical densities according to the formula $K = \frac{C}{D}$ (where K is the conversion factor, C is concentration in milligrams per cent and D is optical density) for that range of concentration where K is constant.

Standardization performed in the preceding manner has yielded a K value of 6.2 for concentrations up to 5 mg. per cent of bilirubin. Thus, Y_{575} times 6.2 equals concentration of bilirubin in milligrams per cent.

SPECTROPHOTOMETRIC STUDIES

In the development of the method, certain spectrophotometric studies were carried out. Bilirubin solutions, icteric urine, and normal urine were treated according to the described method with certain variations which will be noted. The spectral transmittance curves were then determined.* They are reproduced in Figs. 1 to 3.

The preparation of the material for the study was made as follows:

Preparation 1. Bilirubin solution (equivalent to 2 mg. per cent in urine) treated with diazo reagent and not subsequently acidified (*Curve 1*, Fig. 1).

Preparation 2. Bilirubin solution (same as in Preparation 1) treated with diazo reagent and then acidified (*Curve 2*, Fig. 1).

Preparation 3. Normal urine treated with diazo blank† and not subsequently acidified (*Curve 3*, Fig. 2).

Preparation 4. Normal urine treated with diazo reagent and not subsequently acidified (*Curve 4*, Fig. 2).

Preparation 5. Normal urine treated with diazo blank† and then acidified (*Curve 5*, Fig. 2).

Preparation 6. Normal urine treated with diazo reagent and then acidified (*Curve 6*, Fig. 2).

Preparation 7. Icteric urine treated with diazo blank† and not subsequently acidified (*Curve 7*, Fig. 3).

Preparation 8. Icteric urine treated with diazo reagent and not subsequently acidified (*Curve 8*, Fig. 3).

Preparation 9. Icteric urine treated with diazo blank† and then acidified (*Curve 9*, Fig. 3).

*On a General Electric recording spectrophotometer at the Stamford Research Laboratories of the American Cyanamid Co.

†Hydrochloric acid, 1.5 per cent (15 ml. concentrated hydrochloric acid in 1,000 ml. solution).

Preparation 9. Icteric urine treated with diazo blank† and then acidified (*Curve 10*, Fig. 3).

Curve 1 represents the spectral transmittance of the red (nonacidified) azobilirubin, the derivative commonly employed for quantitative bilirubin determinations in serum. This red pigment has its absorption maximum at 530 to 540 millimicrons. If the red azobilirubin is strongly acidified with hydrochloric acid, it is at once converted to a purple pigment.* *Curve 2* shows the spectral transmittance of this pigment. The purple (acidified) azobilirubin has

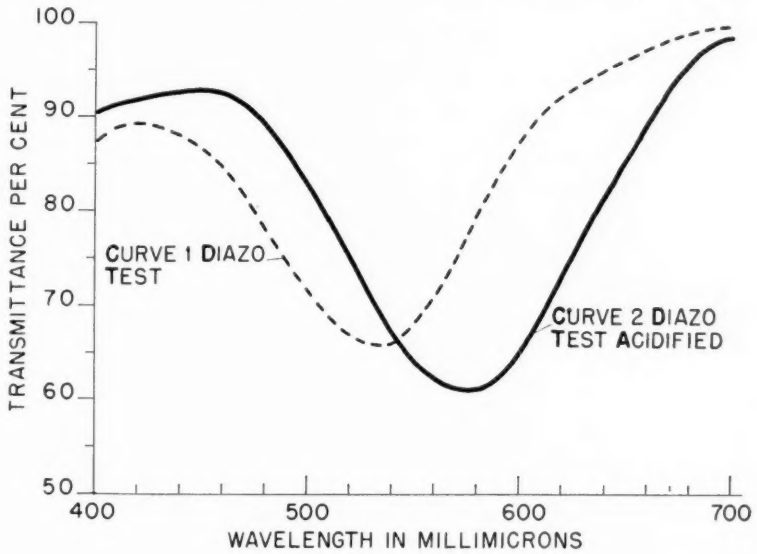

Fig. 1.

its absorption maximum at 570 to 580 millimicrons. Since *Curves 1* and *2* represent equal amounts of bilirubin, it is seen that the purple pigment absorbs more light at 575 mμ than the red pigment at 535 millimicrons. This alone suggests that the purple (acidified) azobilirubin is the more sensitive derivative for photometric analysis.

Fig. 2 presents the curves obtained using normal urine, presumably bilirubin free. *Curve 4* shows the transmittance of a pigment developed by diazotization, and *Curve 6* shows the change in transmittance produced upon acidification. *Curve 3* is the blank for *Curve 4* and *Curve 5* is the blank for *Curve 6*.

Curve 4 has no maximum absorption band in the visible spectrum, while *Curve 6* has a broad maximum absorption band from 440 to 480 millimicrons. Both preparations show significant absorption at the wave lengths at which azobilirubin may be read, that is, 530 mμ for the red or 575 mμ for the purple pigment. The presence of the interfering substances in normal urine clearly indicates the need for a means of correction in methods employing direct diazotization. For convenience we designate this interfering material as x-chromogen.

*The conversion of red azobilirubin to a purple pigment by acidification with hydrochloric acid has been known for some time and, in fact, is attributed to both Ehrlich and Proscher (original reference not available) as cited by Müller.[10]

Fig. 3 presents the transmittance curves of icteric urine treated similarly to the normal urines of Fig. 2. *Curve 8* represents the transmittance characteristics of red azobilirubin modified by the presence of the unacidified diazo-

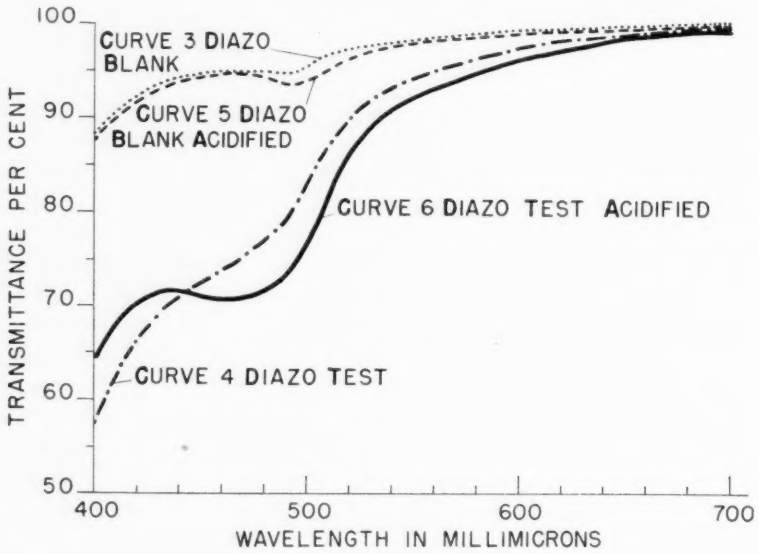

Fig. 2.

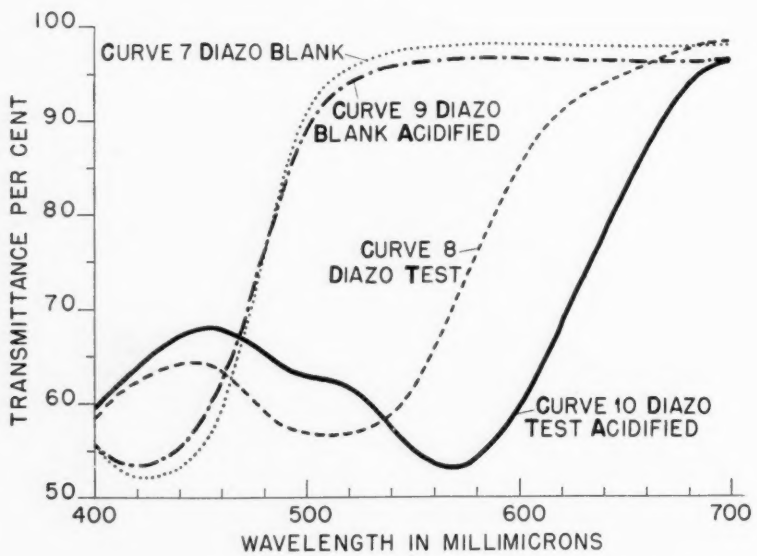

Fig. 3.

tized x-chromogen. This shows a shift of the absorption maximum from 530 to 540 mμ for pure bilirubin (*Curve 1*, Fig. 1) to 490 to 530 millimicrons. This curve is smooth since x-chromogen has no absorption maximum in the visible

spectrum. *Curve 7* is the blank for *Curve 8*. *Curve 10* presents the transmittance characteristics of purple (acidified) azobilirubin (*Curve 2*, Fig. 1) modified by the presence of acidified diazotized x-chromogen. *Curve 10* shows that the absorption maximum is neither shifted nor significantly broadened, but there is a distinct depression in the otherwise smooth curve at 490 to 510 mμ, apparently the effect of the absorption due to acidified diazotized x-chromogen. These properties emphasize the superiority of the purple (acidified) azobilirubin for photometric analysis. *Curve 9* is the blank for *Curve 10*.

DERIVATION AND APPLICATION OF CORRECTION EQUATION

The general form of the equation which applies for the photometric determination of the individual pigments of a two component mixture is derived as follows.

Let: X_a = optical density of X-substance at wave length a.
X_b = optical density of X-substance at wave length b.
Y_a = optical density of Y-substance at wave length a.
Y_b = optical density of Y-substance at wave length b.
M_a = optical density of a mixture of X and Y at wave length a.
M_b = optical density of a mixture of X and Y at wave length b.

$$(1)\quad R_x = \frac{X_a}{X_b} \qquad (2)\quad R_y = \frac{Y_a}{Y_b}$$

If substances X and Y do not react chemically with one another, the following holds:

$$(3)\quad M_a = X_a + Y_a \qquad (4)\quad M_b = X_b + Y_b$$

From (2) and (3):

$$(5)\quad M_a = X_a + R_y Y_b$$

And from (1) and (4):

$$(6)\quad M_b = \frac{X_a}{R_x} + Y_b$$

Solving equations (5) and (6) for Y_b we obtain:

$$(7)\quad Y_b = \frac{R_x M_b - M_a}{R_x - R_y}$$

An inspection of the spectral transmittance curves in Figs. 1 to 3 indicates two suitable wave lengths are 575 and 450 millimicrons. If we let Y represent bilirubin; X, the interfering chromogen; a, 450 mμ; and b, 575 mμ, then equation (7) can be rewritten:

$$(8)\quad Y_{575} = \frac{R_x M_{575} - M_{450}}{R_x - R_y}$$

To apply the formula for use, the numerical values of R_x and R_y at 450 and 575 mμ must be derived. Values for R_x were obtained by diazotizing normal, bilirubin-free urines by the method given previously and finding the optical densities at 450 and 575 millimicrons.

$$R_x = \frac{X_{450}}{X_{575}} = \frac{\text{optical density of x-chromogen at 450 m}\mu}{\text{optical density of x-chromogen at 575 m}\mu}$$

TABLE I. DETERMINATION OF EQUATION CONSTANTS

	NUMBER OF DETERMINATIONS	MEAN VALUE	STANDARD DEVIATION OF MEAN
$R_x = \frac{X_{450}}{X_{575}}$	49	5.19	± .537
$R_y = \frac{Y_{450}}{Y_{575}}$	61	0.25	± .066

TABLE II. ADDITION OF BILIRUBIN TO URINE

CASE	M_{575} OBSERVED	M_{450} OBSERVED	Y_{575} COMPUTED	Y_{575} ADDED	$\triangle Y_{575}$* COLUMN 4 MINUS COLUMN 5
1	.125	.377	.055	.043	+ .012
2	.194	.432	.117	.102	+ .015
3	.324	.475	.234	.222	+ .012
4	.083	.187	.049	.051	− .002
5	.131	.426	.051	.051	0
6	.111	.328	.051	.051	0
7	.161	.420	.084	.084	0
8	.155	.367	.089	.084	+ .005
9	.212	.758	.070	.084	− .014
10	.215	.620	.101	.110	− .009
11	.177	.420	.101	.110	− .009
12	.229	.783	.082	.110	− .028
13	.149	.177	.120	.116	+ .004
14	.146	.177	.117	.116	+ .001
15	.155	.187	.125	.116	+ .009
16	.155	.229	.117	.116	+ .001
17	.161	.229	.123	.116	+ .007
18	.149	.116	.133	.116	+ .017
19	.155	.215	.120	.116	+ .004
20	.158	.237	.118	.116	+ .002
21	.071	.177	.039	.037	+ .002
22	.105	.347	.040	.037	+ .003
23	.066	.155	.038	.037	+ .001
24	.056	.116	.036	.037	− .001
25	.076	.248	.030	.037	− .007
26	.071	.208	.033	.037	− .004
27	.076	.237	.032	.037	− .005
28	.111	.181	.080	.070	+ .010
29	.137	.337	.076	.070	+ .006
30	.111	.168	.083	.070	+ .013
31	.108	.131	.087	.070	+ .017
32	.111	.244	.068	.070	− .002
33	.111	.222	.072	.070	+ .002
34	.119	.244	.076	.070	+ .006
35	.097	.184	.065	.077	− .012
36	.111	.229	.071	.077	− .006
37	.128	.347	.064	.077	− .013
38	.197	.276	.151	.158	− .007
39	.190	.240	.151	.158	− .007
40	.197	.284	.150	.158	− .008
41	.194	.276	.148	.158	− .010
42	.208	.382	.141	.158	− .017
43	.347	.284	.307	.323	− .016
44	.342	.218	.316	.323	− .007
45	.357	.342	.306	.323	− .017
46	.347	.201	.323	.323	0
47	.347	.256	.311	.323	− .012

*$\triangle Y_{575}$. The computed minus the expected optical density of the added amount of bilirubin, that is, column four minus column five.

Summary of data in Table II: Mean deviation ($\triangle Y_{575}$) regardless of sign, .0075. Range of error expressed as bilirubin, −.17 to +.11 mg. per cent. Average per cent recovery of added bilirubin, 99.7 per cent.

Values for R_y were similarly determined with pure bilirubin solutions. Table I shows the summarized data for the determinations of R_x and R_y.

Since $R_x = 5.19$ and $R_y = 0.25$, substitution of these values in equation (8) gives the final equation:

$$(9) \quad Y_{575} = \frac{5.19\ M_{575} - M_{450}}{5.19 - .25} = 1.05M_{575} - .202M_{450}$$

where Y_{575} equals the optical density of the bilirubin component of the mixture at 575 mμ, M_{575} equals the optical density of the mixture of bilirubin and x-chromogen at 575 mμ, and M_{450} equals the optical density of the same mixture at 450 millimicrons.

To determine the reliability of the equation and the method, recoveries of bilirubin added to normal (bilirubin-free) urine were carried out. The computed optical densities in these mixtures (Y_{575}) were compared with the optical densities of the same amounts of pure bilirubin determined simultaneously. Since precise amounts of bilirubin cannot be added conveniently to urine directly, the additions were accomplished by dissolving the desired amount of bilirubin in the alcohol used as the diluent in the method.

Table II presents a summary of these data.

The differences between the calculated and expected optical densities (Y_{575} of Table II) were small and were random with respect to sign. The average recovery was 99.7 per cent. We conclude that equation (9) introduces no significant theoretical error and is therefore valid and applicable for the calculation of the optical density of bilirubin mixed with x-chromogen according to our method.

The arithmetic mean of the optical density differences (column six, Table II) was 0.0075 regardless of sign, and this optical density is equivalent to approximately 0.04 mg. per cent of bilirubin. An error of this order of magnitude is of little significance.

In order to show the order of magnitude of x-chromogen we have presented in Table III data obtained from a representative group of icteric urines. These results show that x-chromogen was encountered in all of

TABLE III. RELATIVE PROPORTIONS OF BILIRUBIN AND X-CHROMOGEN IN ICTERIC URINE

CASE	DILUTION FACTOR	OBSERVED		COMPUTED		RELATIVE PROPORTION OF X AND Y (%)		EXPRESSED AS MG. % BILIRUBIN	
		M_{450}	M_{575}	X_{575}*	Y_{575}†	X	Y	X-CHROMOGEN X	TRUE BILIRUBIN Y
1	5	.240	.362	.030	.332	8	92	.93	10.30
2	2	.523	.721	.072	.649	10	90	.90	8.04
3	1	.357	.377	.053	.324	14	86	.33	2.01
4	5	.248	.205	.040	.165	20	80	1.25	5.10
5	1	.796	.577	.132	.445	23	77	.82	2.76
6	1	.638	.367	.110	.257	30	70	.68	1.59
7	1	.409	.174	.074	.100	43	57	.46	.62
8	1	.319	.119	.058	.061	49	51	.36	.38
9	1	.367	.119	.068	.051	57	43	.42	.32
10	1	.387	.111	.072	.039	65	35	.45	.24

*$X_{575} = M_{575} - Y_{575}$.

†Y_{575} computed from equation (9).

these specimens and in amounts accounting for from 8 to 65 per cent of the observed optical density at 575 millimicrons. It is evident from these data that the correction for x-chromogen is essential for the true bilirubin values.

MISCELLANEOUS DETAILS OF TECHNIQUE

Certain points of technique were investigated to determine conditions for optimum color development and reproducibility. The choice of alcohol as a diluent was based on the fact that the substitution of water for 95 per cent ethanol in the described method results in a diminution of optical density of diazotized icteric urine at both 450 and 575 millimicrons. An example is given in Table IV.

TABLE IV. WATER VS. 95 PER CENT ETHANOL AS THE DILUENT

DILUENT	OPTICAL DENSITY	
	575 mμ	450 mμ
Distilled water	.268	.168
95% Ethanol	.337	.252

The prescribed order of addition of reagents was found superior to other orders tried. Data in Table V show that each modification resulted in reduction of optical density at 575 millimicrons.

In the described method, thirty minutes are allowed for color development before the addition of acid because the color reaction changes little after

TABLE V. ORDER OF ADDITION OF REAGENTS

ORDER OF ADDITION	OPTICAL DENSITY (575 mμ)		
	SOLUTION 1	SOLUTION 2	SOLUTION 3
1, 2, 3, 4, 5	.350	--	--
1, 3, 4, 2, 5	.310	--	--
1, 2, 3, 4, 5	--	.314	.633
1, 2, 3, 5, 4	--	.233	.475

1, Urine (or, in case of solutions 2 and 3, bilirubin in 95 per cent ethanol).
2, 95 per cent ethanol.
3, Diazo reagent.
4, Color development period (thirty minutes).
5, Concentrated hydrochloric acid, 0.25 milliliter.

twenty-five minutes and is maximal at thirty minutes. Although the purple (acidified) azobilirubin is quite stable, there is no advantage in delaying the reading. The data concerning the rate of color development and the stability of the color are shown in Table VI. Pure bilirubin was used for these determinations.

TABLE VI. RATE OF COLOR DEVELOPMENT

Minutes after addition of diazo reagent	5	10	15	20	25	30	35	45	75
Minutes after addition of acid*							5	15	45
Optical Density	READ AT 530 mμ						READ AT 575 mμ		
Solution 1	.187	.211	.229	.240	.250	.256	.315	.315	.319
Solution 2	.337	.417	.450	.473	.493	.500	.633	.633	.638

*Acid added thirty minutes after diazo reagent.

In order to avoid variations due to rate of urine flow and to obviate turbidities in concentrated specimens, it was found desirable to collect the urine over a timed period. The urine is then diluted to a total volume equal to 100 ml. per hour. After mixing, 1 ml. of this diluted specimen is used for analysis. This dilution obviates turbidities and makes milligrams per 100 ml. equivalent to milligrams per hour. For urine flow above 100 ml. per hour, appropriate correction must be made if the results are to be expressed in units of time. If the urine is turbid even after this dilution, a portion should be centrifuged before taking the 1 ml. for analysis. Highly icteric urines will require even greater dilutions than 100 ml. per hour. Correction must be made for the further dilution in calculating the concentration of bilirubin or for expressing the amount of bilirubin per unit time.

Collection of specimens in stoppered, brown bottles and prompt analysis are recommended to avoid loss of bilirubin as reported by With.[3] We have found losses of 14, 15, and 50 per cent of bilirubin from three icteric urine specimens held twenty-four hours in the refrigerator in stoppered brown bottles.

INTERFERING MATERIALS

We have investigated the possible interference with the procedure by certain common medications. Urine specimens were collected from patients receiving Solu B* and penicillin. Neither of these substances was found to have interfering properties as shown by the Y_{575} values presented in Table VII, Groups I and II.

Sulfathiazole and sulfadiazine in amounts of 25 mg. per cent do not interfere with the test as shown by the Y_{575} values in Table VII, Group III.

TABLE VII. EFFECT OF MISCELLANEOUS MEDICATIONS

TYPE OF SPECIMEN		M_{450}	M_{575}	Y_{575}* COMPUTED
Group I:	Specimens from patients receiving penicillin			
	1	.319	.063	+ .002
	2	.495	.086	− .010
	3	.161	.0315	0
	4	.149	.032	+ .004
Group II:	Specimens from patients receiving Solu B			
	1	.174	.036	+ .003
	2	.161	.032	+ .001
	3	.161	.032	+ .001
Group III:	Normal urine and sulfa compounds			
	Normal urine	.222	.046	+ .003
	Same urine + 25 mg. % Na sulfathiazole	.225	.046	+ .003
	Same urine + 25 mg. % Na sulfadiazine	.225	.046	+ .003
Group IV:	Normal urine and Atabrine			
	Normal urine	.194	.0385	+ .002
	Same urine + 1 mg. % Atabrine	.218	.0385	− .003
	Same urine + 2 mg. % Atabrine	.244	.0410	− .006
	Same urine + 3 mg. % Atabrine	.268	.0435	− .008

Range of error expressed as bilirubin: − .06 to + .02 mg. per cent.
*Y_{575} expected: none.

*This proprietary vitamin B complex for parenteral use contains thiamine, riboflavin, pyridoxine, pantothenate, and nicotinamide.

Atabrine (quinacrine hydrochloride in amounts of 1, 2, and 3 mg. per cent) shows a slight interference as demonstrated in the data of Table VII, Group IV. The 3 mg. per cent concentration would mask approximately 0.05 mg. per cent of bilirubin, an amount we consider insignificant.

Protein in the urine in amounts greater than 0.1 mg. per cent (2 plus ring or acetic acid heat test) causes turbidities which render this method unsuitable. Since most of the commonly used protein precipitants may remove bilirubin with the protein, their use is contraindicated.

In the course of the derivation of the numerial value for R_x, a few urine specimens (four out of fifty-five) were encountered which gave R_x values exceeding the mean of 5.19 by about four times the standard deviation. It is safe to assume that these urine specimens contained some interfering material in addition to x-chromogen. Such an interfering material in an icteric urine would tend to mask a portion of the bilirubin. This effect was observed when two of these urine specimens were used for recovery experiments. In both instances the computed values of Y_{575} were less than expected by an amount corresponding to approximately 0.2 mg. per cent of bilirubin. We have been unable to identify the material responsible for this interference.

ADAPTATION OF METHOD TO FILTER PHOTOMETER

The method presented here would have limited clinical application if it could not be adapted to filter photometers. Its adaptation to one filter photometer was easily accomplished in our laboratory.*

Following the exact procedure outlined under Derivation and Application of Correction Equation, a group of ten normal urine specimens and a series of bilirubin solutions ranging from 0.5 to 4 mg. per cent were diazotized and read in the Lumetron instrument using orange (580 mμ) and blue (420 mμ) filters. From these readings the constants R_x and R_y were calculated and found to be 8.0 and 0.31, respectively. Substituting these constants in equation (8), it becomes: (9_a) $Y_{orange} = 1.04\ M_{orange} - 0.13\ M_{blue}$ where the symbols are analogous to those of equation (9) for the Coleman instrument.

TABLE VIII. ADDITION OF BILIRUBIN TO URINE; RECOVERY ON A LUMETRON PHOTOMETER

CASE	M_{orange} OBSERVED	M_{blue} OBSERVED	Y_{orange} COMPUTED	Y_{orange} ADDED	Y_{orange} COLUMN 4 MINUS COLUMN 5
38	.149	.252	.122	.125	– .003
39	.146	.215	.124	.125	– .001
40	.149	.260	.121	.125	– .004
41	.143	.248	.117	.125	– .008
42	.152	.337	.114	.125	– .011
43	.260	.244	.238	.252	– .014
44	.260	.194	.245	.252	– .007
45	.268	.306	.238	.252	– .014
46	.264	.178	.249	.252	– .003
47	.260	.222	.241	.252	– .011

Mean deviation regardless of sign: .0076.
Range of error expressed as bilirubin: – .008 to – 0.11 mg. per cent.
Average per cent recovery of added bilirubin: 96 per cent.

*A Lumetron photometer, model 400A, was employed.

A calibration was carried out using the procedure outlined under Method. A conversion factor (K) of 8.1 was obtained which was constant up to the 4 mg. per cent concentration. Thus 8.1 times Y_{orange} equals milligrams per cent bilirubin.

The validity of equation (9_a) was tested with mixtures of normal urine and bilirubin as described (Derivation and Application of Correction Equation). The data of this experiment are shown in Table VIII. The recoveries appear to be entirely satisfactory.

As an additional check on the reliability of the method using the filter photometer, a parallel series of determinations were done on icteric urine specimens using both instruments. The results show close agreement, as can be seen in the data of Table IX.

TABLE IX. BILIRUBIN IN ICTERIC URINE; DETERMINED SIMULTANEOUSLY ON TWO PHOTOMETERS

.19 TO .99 MG. %		1.00 MG. % AND OVER	
COLEMAN	LUMETRON	COLEMAN	LUMETRON
.19	.23	1.04	1.15
.20	.27	1.12	1.22
.22	.28	1.21	1.32
.22	.36	1.26	1.29
.23	.27	1.45	1.56
.24	.29	1.61	1.73
.25	.26	1.68	1.75
.28	.36	1.69	1.84
.30	.30	1.77	1.93
.35	.36	1.95	2.00
.36	.36	2.01	1.92
.38	.28	2.18	2.32
.38	.36	2.28	2.28
.39	.45	2.28	2.30
.40	.53	2.30	2.30
.41	.49	2.37	2.54
.47	.62	2.40	2.44
.48	.67	2.60	2.68
.52	.68	2.62	2.60
.55	.63	2.72	2.70
.69	.79	2.74	2.72
.70	.84	2.79	2.84
.80	.89	2.94	3.00
		3.01	3.04
		3.35	3.20

DISCUSSION

The method for the determination of bilirubin in urine which we have presented here is essentially a modification or adaptation of the Ehrlich diazo reaction, as employed in the Malloy and Evelyn[8] method for serum bilirubin. Since normal or icteric serum has no chromogen other than bilirubin which upon diazotization produces a significant amount of color with a spectral absorption maximum at 530 mμ, no separation of the bilirubin from the serum is necessary. This makes the determination in serum relatively simple and straightforward.

However, in normal or icteric urine there are amounts of chromogenic material which will react with the diazo reagent to cause a significant inter-

ference with the photometric reading of the diazotized bilirubin. Therefore in analyzing urine for bilirubin by this reaction one is faced with the necessity of (1) separating the bilirubin or its colored derivative from the urine by chemical or physical means, or (2) determining and applying a correction for the interfering material. Attempts at applying the first alternative have so far, to the best of our knowledge, proved unsuccessful. In the present work we have succeeded in employing the second alternative. The correction equation which we have derived was tested by adding known amounts of bilirubin to urine and obtaining satisfactory recoveries. This demonstrated to our satisfaction that neither significant theoretic nor systematic errors were encountered in the procedure.

We believe that we have enhanced the specificity and sensitivity of the determination by converting the red azobilirubin with spectral absorption maximum at 530 mμ to the purple azobilirubin with spectral absorption maximum at 575 mμ through acidification with hydrochloric acid. This has two beneficial effects. Our data show that in pure solution the red azobilirubin has less optical density at 530 mμ than an equivalent amount of purple azobilirubin at 575 millimicrons. Thus small amounts of bilirubin can be determined more accurately as the purple derivative than as the red devivative. The second beneficial effect is that at 575 mμ the interference of the nonbilirubin material which we have designated x-chromogen is significantly less than at 530 millimicrons. Even though the principle on which our correction equation is based would be equally valid for the readings at 530 mμ, nevertheless any means of minimizing the effect of the interfering material should be utilized.

With respect to the sensitivity of the method, we estimate a likely error of .015 in the computed values of Y_{575}. This is twice the mean error of our addition and recovery experiments (Table II). Such an error is equivalent to approximately 0.1 mg. per cent of bilirubin in urine. In certain instances where two- or threefold dilutions of the urine have been made, the error might be as large as 0.2 to 0.3 mg. per cent in terms of bilirubin concentration in the original urine. We feel that this is a liberal estimate of the lower limit of sensitivity of the method. In fact, repeated values of as little as 0.2 mg. per hour have been found to date only in patients having disorders of bilirubin metabolism.

We believe that diazotization provides a more specific color reaction for bilirubin than oxidation methods using ferric salts or nitric acid as in the method of Singer and Kubin.[9] The red, blue, and green colored oxidation products are not too well characterized.

Finally, we wish to emphasize the relative simplicity of the procedure. It requires no special apparatus other than a reliable clinical photometer. The required reagents are readily available and easy to prepare. Once the correction equation and the standard curve or calibration factor have been determined for the instrument to be used, the routine performance of the test requires practically no more manipulation than a serum bilirubin determination.

SUMMARY

A method for the quantitative determination of bilirubin in urine is presented. It employs the principle of direct diazotization of urine with Ehrlich's diazo reagent.

A correction equation is derived and applied for the elimination of nonbilirubin interfering material.

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A PRACTICAL METHOD FOR THE PREPARATION OF ORGANS FOR THE DETERMINATION OF ANTIBIOTIC CONTENT

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IN THE course of our studies on penicillin tissue levels in small laboratory animals (rats and mice) we were faced with the problem of rapidly emulsifying a large number of organ samples under sterile conditions. The small tissue volumes, their large number, and the time and expense involved precluded the use of the semimicro Waring blender or of mortar and pestle.

We solved this problem by using a Foredom Lighter Duty Flexible Shaft machine (approximately 1/25 horsepower), fitted with a *wire brush*, and ½, 1, and 2 ounce jars with metal screw caps. (Fig. 1.) Holes just large enough to accommodate the shaft of the wire brush were made in the covers of the jars. The holes were plugged with cotton and the covered jars were sterilized. The tissues—lung, liver, kidney, and spleen—were removed aseptically and placed in jars appropriate to the volume of the sample. Two volumes of sterile 1 per cent phosphate buffer (pH 6.0) and some sterile alundum were added. The sterilized wire brushes were inserted through the jar caps and attached to the handpiece. Rotation of the wire brush at approximately 10,000 revolutions per minute produced a finely ground tissue mash in about one minute. This mash was poured into centrifuge tubes and spun at low speed (500 to 1000 revolutions per minute); the clear supernatant liquid was used for assay. Used brushes were washed in 20 per cent NaOH to remove grit and tissue particles, rinsed in tap water, and resterilized.

Eighty to one hundred tissue samples, ranging from 0.5 to 30 Gm. each, can be prepared satisfactorily in a single day by this method.

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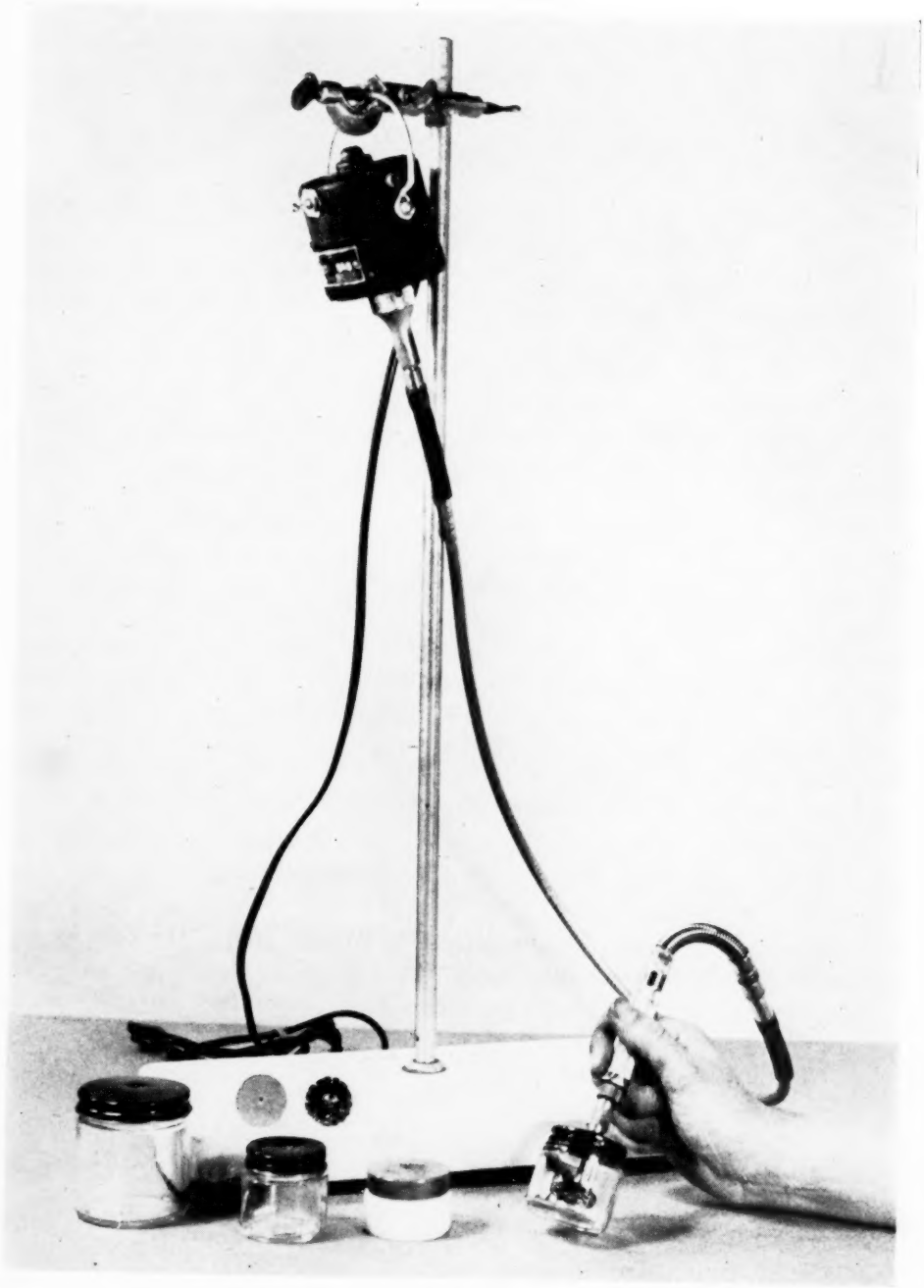

Fig. 1.

A RAPID METHOD FOR SERUM CALCIUM DETERMINATION

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IN MANY clinical situations, such as convulsions due to infantile tetany, a rapid method for determination of serum calcium, even if somewhat less accurate, would be more valuable to clinicians than the time-consuming standard method. A method to be most useful should possess speed and a reasonable degree of accuracy and require a minimum amount of blood.

The method to be described is a nephelometric procedure adapted from the one for determination of calcium in water.[1] Although less precise than a standard micromethod, the procedure can attain an accuracy adequate for the usual clinical purposes; it takes less than an hour and requires only 0.5 ml. of serum.

Potassium oleate reagent in Duponol solution reacts with calcium ion in ammoniacal solution to give a white colloidal suspension of calcium oleate. The degree of turbidity is proportional to the amount of calcium present in trichloroacetic acid filtrate of serum. The turbidity is measured in a photoelectric colorimeter. The method described is adapted to Klett-Summerson (micro) photoelectric colorimeter. Most of the common ions normally present in human serum have no effect on the reaction. Duponol prevents the formation of magnesium oleate, thus eliminating interference due to this ion.

REAGENTS AND APPARATUS

Potassium Oleate Reagent.—This reagent is prepared as described by Saifer and Clark.[1] Shake 7.05 Gm. of oleic acid* with a solution of 1.60 Gm. of potassium hydroxide in 5.0 ml. of distilled water. Transfer the emulsion by means of 50 ml. of 70 per cent ethanol to a flask. Reflux the mixture for one hour and dilute with distilled water to 250 ml. in a volumetric flask.

Duponol Solution.—Prepare 3 per cent solution in distilled water. Duponol P. C. is an emulsifying reagent.†

Potassium Oleate-Duponol Reagent.—To each 100 ml. of Duponol solution add 20 ml. of potassium oleate reagent. Filter off, or remove by centrifugation, any sediment formed. This reagent is stable at room temperature but will come out of solution at lower temperatures. It can be brought back into solution by warming in an incubator at 37° C.

Twenty per cent Trichloroacetic Acid.—Dissolve 10 Gm. of pure trichloroacetic acid in distilled water and make up to 50 milliliters. It is not desirable

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*Eastman Kodak Company, Rochester, N. Y.

†Sold by Dyestuffs Department of E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

to make more at a time because the solution is not completely stable. The solution must be kept in the refrigerator when not in use.

Calcium Standard Solution.—Dissolve 0.5 Gm. of pure calcite (calcium carbonate) in a 500 ml. Erlenmeyer flask with 20 ml. of 1:5 dilute hydrochloric acid, being careful to avoid spattering. Add about 200 ml. of distilled water and boil for a few minutes to drive off the carbon dioxide. Cool to room temperature and transfer quantitatively to a 500 ml. volumetric flask. Make up to volume with carbon dioxide-free distilled water. Store in a glass-stoppered bottle. This standard should be checked for exact calcium content using a permanganate titration procedure. Adjust the concentration of the standard to 40.0 mg. of calcium per 100 milliliters.

Dilute Calcium Standard Solution.—Dilute the preceding standard solution in 100 ml. volumetric flasks. Add from a burette 10.0, 15.0, 20.0, 25.0, and 30.0 ml. of the calcium standard solution and then add carbon dioxide-free distilled water to the mark to get 4, 6, 8, 10, and 12 mg. per 100 ml., respectively.

Pipettes.—Two types of pipettes are used in the method; the constriction type,[2, 3] modified to a larger volume, and syringe pipettes,[4, 5] modified for semiautomatic delivery. The accuracy of a ml. syringe is about 0.01 per cent.[6] The syringe pipettes* are extraordinarily accurate, and furthermore they are extremely convenient to handle. When the same volume of reagent is to be added to a number of samples, these pipettes will save much time. On the other hand, they are not well suited for pipetting many different samples, because the dead-space of these pipettes requires a thorough cleaning between each sample. In this case the constriction pipettes are more convenient. When the composition of the samples does not differ too much, the washing of the pipettes can be neglected. The accuracy of a 1 ml. constriction pipette is about 0.1 per cent.[6]

The sizes of constriction pipettes used are: 0.5 ml. and 1.3 milliliters.† With syringe pipettes it is convenient to have pipettes in the sizes of 1 and 2.0 ml. to deliver 0.5 and 1.0 ml., respectively.

PROCEDURE

Deliver 0.5 ml. of serum into a 12 ml. Pyrex centrifuge tube. Add 1.0 ml. of distilled water (with a 2 ml. syringe pipette adjusted to deliver 1.0 ml.). Then add 0.5 ml. of 20 per cent trichloroacetic acid (with syringe pipette adjusted to deliver this amount). Mix the contents thoroughly with a paraffined wooden applicator and allow it to stand for five minutes. Centrifuge for ten minutes at 2,500 revolutions per minute. Transfer 1.3 ml. of centrifugate (with the constriction pipette) into a calibrated colorimeter tube. Add 0.2 ml. of concentrated ammonium hydroxide.‡ Finally add 1 ml. of potassium oleate-Duponol reagent (with a syringe pipette); allow it to stand for fifteen minutes. Read the turbidity in a Klett-Summerson (micro) photoelectric colorimeter

*The syringe pipette frame may be obtained from Northern Tool and Instrument Company, Flushing, N. Y.

†Constriction pipettes may be obtained from Arthur H. Thomas, Company, Philadelphia, Pa.

‡Another 1 ml. syringe pipette adjusted to deliver 0.2 ml. is recommended as a timesaver.

using a 420 (blue) filter.* Set the zero against the distilled water blank (as described in the Klett-Summerson manual) and then proceed as usual. It has been found most expedient to construct the standard curve every time a specimen is analyzed in order to keep error at a minimum. Treat 0.5 ml. of dilute standard solutions of 6, 8, 10, and 12 mg. of calcium per 100 ml. exactly as the serum would be treated. Record the optical densities, D_s. Read the unknown the same way, D_u. Convert the unknown optical density into milligrams of calcium per 100 ml. of serum according to the equation:

$$\text{Mg. Ca per 100 ml. of serum} = \frac{D_u}{D_s} \times \text{Mg. Ca per 100 ml. of standard solution.}$$

EXPERIMENTAL

Saifer and Clark have investigated various conditions necessary for calcium oleate formation for the determination of calcium in water. The following conditions were studied in this laboratory to insure optimum conditions for calcium oleate formation with trichloroacetic acid filtrate.

Effect of Variation of Potassium Oleate Reagent.—To 1.3 ml. of trichloroacetic acid centrifugate were added 0.5, 1.0, 1.5, and 2.0 ml. of potassium oleate-Duponol reagent, respectively. One milliliter was found to be an adequate amount to obtain the maximum optical density.

Effect of Variation of Ammonium Hydroxide.—To 1.3 ml. trichloroacetic acid centrifugate were added varying amounts of concentrated NH_4OH, and then 1.0 ml. of potassium oleate-Duponol reagent was added. The optical densities observed remained the same from 50 c. mm. of concentrated NH_4OH up to 320 cubic millimeters. However there was a decrease in the optical densities observed when 640 c. mm. or more of ammonium hydroxide were added.

Effect of Variation of Time.—The reaction seems to take place almost instantly. However it has been found that a slight increase in turbidity takes place from fifteen minutes to thirty minutes; thereafter the turbidity remains almost unchanged.

TABLE I. REPRODUCIBILITY OF PRESENT METHOD

SAMPLE	NEPHELOMETRIC METHOD (MG. PER 100 ML.)	DEVIATION FROM AVERAGE (MG. PER 100 ML.)
1	9.7	+0.1
2	9.4	−0.2
3	9.1	−0.5
4	9.8	+0.2
5	9.3	−0.3
6	9.8	+0.2
7	9.8	+0.2
8	9.6	+0.0
9	9.3	−0.3
10	9.8	+0.2
Average	9.6	

*An experiment with the Beckman spectrophotometer has shown that optical density was apparently greater the shorter the wave length. Therefore the blue filter was chosen for the application of the method to the Klett-Summerson instrument.

Precision of the Method.—The results of ten analyses on one sample of human serum are shown in Table I. The average value is 9.6 mg. of calcium per 100 ml., and the maximum deviation from the average is 0.5 mg. per 100 milliliters.

TABLE II. DETERMINATION OF ADDED CALCIUM TO POOLED SERUM

SERUM	KRAMER-TISDALL (MACROMETHOD) 2.0 ML. (MG. PER 100 ML.)	RECOVERY	NEPHELOMETRIC 0.5 ML. (MG. PER 100 ML.)	RECOVERY
S	10.1		10.4	
S + 3.9	14.1	4.0	13.8	3.4
T	10.3		10.7	
T + 3.9	13.8	3.5	14.1	3.4
U	9.9		10.3	
U + 3.9	13.6	3.7	14.1	3.8
V	10.8		9.6	
V + 3.6	14.1	3.3	12.9	3.3

Each determination shown is the average of triplicate.

The Extent of Recovery.—The average recovery for the three experiments when 3.9 mg. of calcium were added to 100 ml. of serum was 3.7 for the Kramer-Tisdall method and 3.5 mg. per 100 ml. of serum for the nephelometric method. In the case of 3.6 mg. per 100 ml. of serum the recoveries were 3.3 and 3.3 mg. per 100 ml. for both the Kramer-Tisdall and the nephelometric methods.

Relationship of Concentration to Optical Density.—Nephelometric measurements are carried out by the same procedure and instruments used for the measurements of substances in solution, that is by comparison against a series of standards. Transmittance measurement, particularly when used in instruments equipped with a photoelectric cell, is the most sensitive and satisfactory. For the procedure described, it has been found that optical density is directly proportional to concentration, and thus resembles optical density for substances in solution. Therefore in this method turbidity measurement is carried out and results are calculated in the same manner as for light absorption.

SUMMARY

A rapid method for the determination of serum calcium has been presented. It is possible to obtain the results in an hour on a duplicate sample of 0.5 milliliter. The results have been found to be adequate for clinical purposes.

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CIRCULATION TIME: AN ACCURATE INDICATOR FOR OBJECTIVE MEASUREMENT

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MANY methods for measuring the velocity of blood have been proposed in recent years. Despite this, there are few that are simple to perform and dependable with reference to accurate reduplication of measurement.

The response to the particular substance used may be subjective, such as the sensation of heat,[1-3] neuromuscular stimulation,[4, 5] or taste,[6, 7] while objective responses may be manifested by their color[8, 9] radioactivity,[10] or fluorescence.[11] Obviously any method depending upon the patients' perception is subject to uncontrollable factors which modify the accuracy and consequently the clinical value of the determination. In addition such procedures are not applicable to the unconscious patient.

During the course of clinical investigations of the state of the circulation in surgical patients with the dye T1824, the arrival of the dye in the vessels of the ear was first studied with the Millikan oximeter.[12] The galvanometer deflection with this instrument was not sufficiently rapid for circulation time measurement, nor was the deflection of sufficient magnitude to produce an unmistakable end point. The photometer to be described gives improved response and deflection. Stability is sufficient to permit valid recording of the changing optical density of T1824 over a considerable period of time. Studies of the mixing time of T1824, using this method, will be reported in a later communication.

The principle involved depends upon the measurement of light intensities which have been adequately filtered to insure relative monochromacy. There are three essential components to the apparatus, namely a stable light source and screening filter which transmit light in the vicinity of 6200A, a photocell and direct coupled amplifier of high sensitivity and good stability, and an indicating galvanometer or graphic recorder.

APPARATUS

Light Source.—The light source is mounted at one end of the ear piece (*A*, Fig. 1) and utilizes a 6 to 8 volt bulb (Mazda 50) operated at 5.5 volts. In addition to supplying the necessary light, the bulb also furnishes gentle heat which produces vasodilatation with consequent increase in both blood and dye thickness. This is desirable since an increase in dye thickness produces a reduction in monochromatic light intensity. The lamp current is maintained at a constant value by the introduction of the countercell (*B9*, Fig. 2) and resistance into the circuit. Constant current is indicated by central position of the galvanometer (*G*, Fig. 2) connected across *R7* (Fig. 2). Voltage regulation is better than 0.25 per cent. A wet storage cell (*D*, Fig. 1) is used to operate the light.

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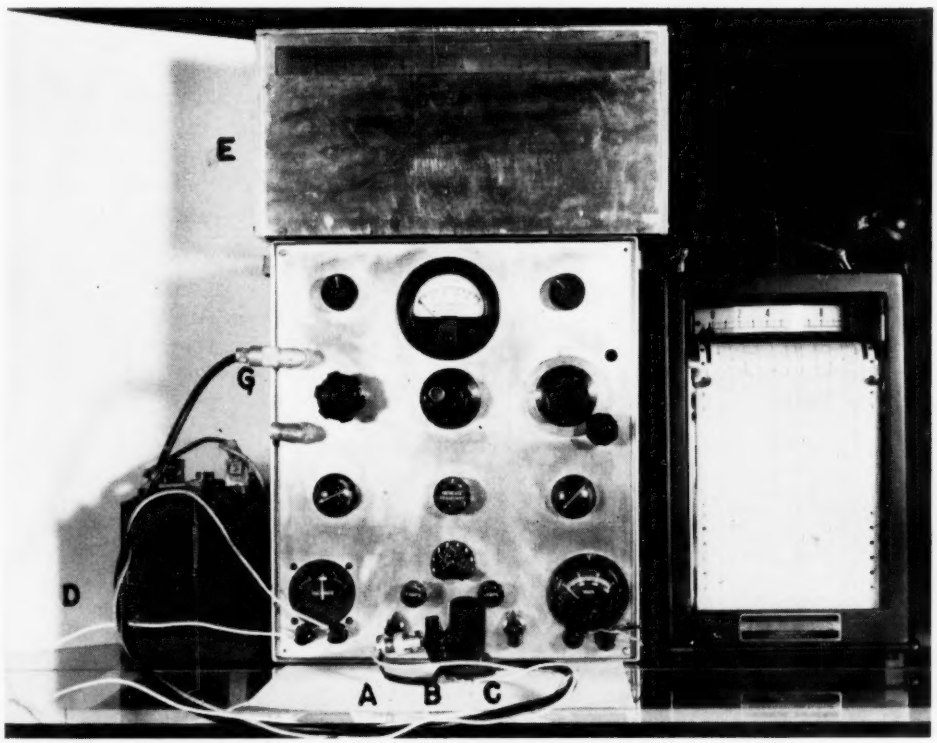

Fig. 1.—Apparatus for recording circulation time with T1824. *A*, Light source; *B*, filter compartment; *C*, photocell compartment; *D*, storage battery for light source; *E*, mirror galvanometer; *F*, Esterline-Angus graphic recording meter; *G*, direct coupled amplifier.

Light Filter.—The screening filter* is a composite unit consisting of two Corning glasses, No. 2418 and No. 9780. The exact spectral transmission, as measured with a spectrophometer, lies between 5880A and 6500A, with 100 per cent transmission at 6100A and 80 per cent at 6200A. The filter is placed in the compartment (*B*, Fig. 1) in front of the photocell and completely screens all light passing through the ear to the photocell.

Photocell.—Photronic cells were not considered applicable because of their inherent instability and sensitivity to infrared light.[13] In addition, the output of photronic cells is insufficient for recording low values of light intensity with relatively rugged instruments. The photoemissive type of cell, R.C.A. No. 926, was found to be completely suitable because of its spectral response, small size, and stability.† Adequate light and capacity shielding are provided by means of a small metal cylinder which is securely fastened to the frame of the ear piece (*C*, Fig. 1). The light aperture is 1 cm. in diameter. The photocell is connected to the amplifier by means of a high insulation shielded cable and coaxial connector. An anode potential of 7.5 to 10 volts will not produce ionization of the residual gas in the photocell. The response to minute changes in light intensity is instantaneous.

Amplifier.—In order to obtain maximum sensitivity plus large response from a phototube, it is necessary that the grid current of the measuring tube be in the vicinity of 10^{-13} amperes. Grid current in measuring tubes places a lower limit on the current sensitivity. These characteristics may be obtained in specially designed electrometer tubes‡ or in acorn tubes operated under special conditions. Gabus and Poole[14] and recently Nielsen[15] have shown that acorn tubes may be operated with grid current comparable to that

*Light filter No. 620, obtained from Rubicon Company, Philadelphia, Pa.

†Phototubes, R.C.A. Manufacturing Company, Inc., Camden, N. J., Form PT-20R1.

‡FP, 54, General Electric Company, Schenectady, N. Y.; D-96475, Western Electric Company, Newark, N. J.; VW 41, Victoreen Instrument Company, Cleveland, Ohio.

obtained with special electrometer tubes by simply reversing the suppressor and control grids and reducing operating potentials. By virtue of the two positively charged grids, G1 and G2, ions formed in the hot cathode are prevented from reaching the control grid. This eliminates the greatest source of grid current. The electrons, on the other hand, are accelerated toward the control grid, G3.

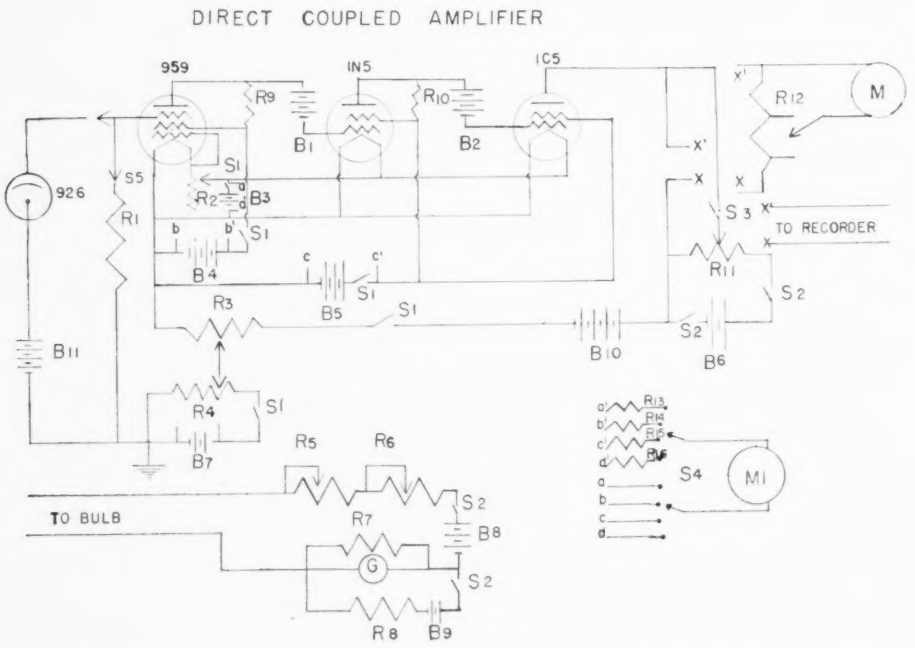

Fig. 2.—Schematic diagram of amplifier. *R1*, 100 to 10,000 megohms; *R2*, 10 ohm wire-wound potentiometer General Radio #301; *R3*, 2,000 ohm wire-wound potentiometer General Radio #301; *R3* 2,000 ohm wire-wound potentiometer General Radio #314 A; *R4*, 20,000 ohm wire-wound potentiometer General Radio #314 A; *R5*, 1 ohm rheostat with 0.25 ohm shunt; *R6*, 5.0 ohm rheostat with 1.0 ohm shunt; *R7*, 10 ohm wire-wound fixed resistor, low temperature coefficient; *R8*, 5.0 ohm semivariable, wire-wound resistor; *R9*, 1.0 megohm wire-wound fixed resistor; *R10*, 1.0 megohm wire-wound fixed resistor; *R11*, 10,000 ohm wire potentiometer; *R12*, 20,000 ohm wire-wound voltage divider; *R13*, *R14*, 6,000 ohm wire-wound fixed resistor; *R15*, *R16*, 60,000 ohm wire-wound fixed resistor; *M*, 0 to 20 microampere panel meter with 0 to 100 scale; *M1*, 0 to 1 milliampere panel meter with 0 to 6, 0 to 60 scale; *S1*, *S2*, 4 pole, single throw switch; *S3*, Single pole, single throw switch; *S4*, 2 pole, 4 position selector switch; *B1*, 4.5 volt battery; *B2*, 22.5 volt battery (Minimax); *B3*, 2.0 volt storage cell; *B4*, 6.0 volt battery; *B5*, 45.0 volt battery; *B6*, 3.00 volt battery; *B7*, 6.0 volt battery; *B8*, 6 volt storage battery; *B9*, 2 volt storage cell; *B10*, 22.5 volt battery; *B11*, 7.5 volt battery.

The sensitivity ordinarily obtainable with the special electrometer tube is limited by the stability of the tube itself and that of the batteries.[16] In addition, these tubes require the use of sensitive, long-period galvanometers and cumbersome compensating circuits. These restrictions to single tube circuits make them unsuitable for biologic work, especially when one desires to record a rapidly changing sequence of events.

The amplifier discussed in this paper employs an acorn tube operating in electrometer fashion in conjunction with a current amplifier (Fig. 2). This combination takes advantage of the high sensitivity obtainable with an acorn tube and permits the use of a rugged recording instrument. Inverse feedback further increases the stability of the amplifier and renders it completely free from bothersome drift and fluctuation.[17] The drift over a three-hour test period is less than 0.20 per cent of full-scale deflection. The indicating galvanometer may be any rugged instrument with a minimum full-scale sensitivity of 5 milliamperes. A specially designed, multiple reflection type of galvanometer box (*E*, Fig. 1)

with a mirror galvanometer* movement #3070 and a 12-inch light scale affords unmistakable reading of light intensity. The enclosed light source consists of a pen-light type of bulb, a condensing lens, and a 1.5 volt dry battery. The shunt resistance is equal to the critical damping resistance of the movement. An Esterline-Angus graphic recorder (*F*, Fig. 1) with a 1 Ma. movement may be used for continuous recording of optical density. Full-scale deflection of the recorder is easily obtainable with the standard amount of light passing through an ear of normal thickness and the light filter.

A type R.C.A. #959 acorn tube is used in the first stage. The grid input resistance is selected by means of switch *S5* (Fig. 2). Surface leakage in the grid selector switch is reduced to a minimum by first treating the switch with DC 200† and fusing at a temperature of 300° C. for at least two hours. The suppressor grid, G3, and control grid, G1, are reversed in position. The plate and screen are operated at 6 volts and the filaments at 0.8 volt. These reduced operating conditions improve stability and reduce grid current. A negative bias of 4.5 volts on the control grid G3 results in a linear response. The potential of Grid G1 is maintained at approximately 0.8 volt positive, depending upon its effect on the slope and the linearity of the plate curve.

The second and third stage function as voltage and current amplifiers respectively. The output of the acorn tube, R.C.A. #959, is fed directly to the grid of an R.C.A. #1N5. A negative bias of 4.5 volts is sufficient to control plate current. The screen grid and plate are both operated at 45 volts. An R.C.A. #1C5 is used in the last stage with 45 volts applied to the screen and 22.5 volts to the plate. A negative grid bias of 22.5 volts will permit cutoff operations in this stage.

An input resistance of 10^8 ohms will produce full-scale deflection of the recording meter when the photocell receives light passing through an ear of normal thickness and the #620 filter. A 1 volt input signal will produce a 5 Ma. increase in the plate current of the final stage.

The entire amplifier, with the exception of the photocell, is enclosed in a heavy aluminum cabinet (*G*, Fig. 1). The tubes and input resistances are firmly supported on the metal panel by means of metal brackets and are further enclosed in an airtight plastic case containing anhydrous calcium chloride. This further reduces atmospheric effects on the surface resistance of the tubes and improves stability. All batteries, with the exception of the 6-volt storage cell used for the light source, are enclosed in the cabinet. The B batteries are of medium size since the current drain is minimal.

Operation.—A double pole, double throw switch permits the use of the internal galvanometer, (*M*, Fig. 2) together with the variable shunt (*R12*, Fig. 2) or an external indicating device. The amplifier is placed in operation by means of switch *S1* (Fig. 2). A warm-up of about five minutes is sufficient to insure stability. The bulb and bucking circuit (*B6* and *R11*, Fig. 2) are placed in operation by means of *S2* (Fig. 2). The bucking circuit is necessary only if the indicating meter goes off scale when using the standard amount of illumination. Zero setting of the indicating galvanometer is determined by the position of *R4* (Fig. 2). With the amplifier operating slightly above zero bias, the inverse-feedback control (*R3*, Fig. 2) is gradually rotated clockwise to the point where the meter deflection begins to decrease. This position indicates adequate locking of the input and output signals and effective feedback operation. Further adjustment of the inverse-feedback and zero controls is not necessary. The condition of all batteries may be determined from the meter (*M1*, Fig. 2) by rotating switch *S4* (Fig. 2).

METHOD

The amplifier is placed in operation. No adjustments of zero or feedback controls are necessary. The patient is placed in a recumbent position. The right arm is elevated to the approximate position of the right auricle

*Rubicon Company.

†Liquid Dimethylsilicone, DC 200, supplied by the Dow Corning Corporation, Midland, Mich.

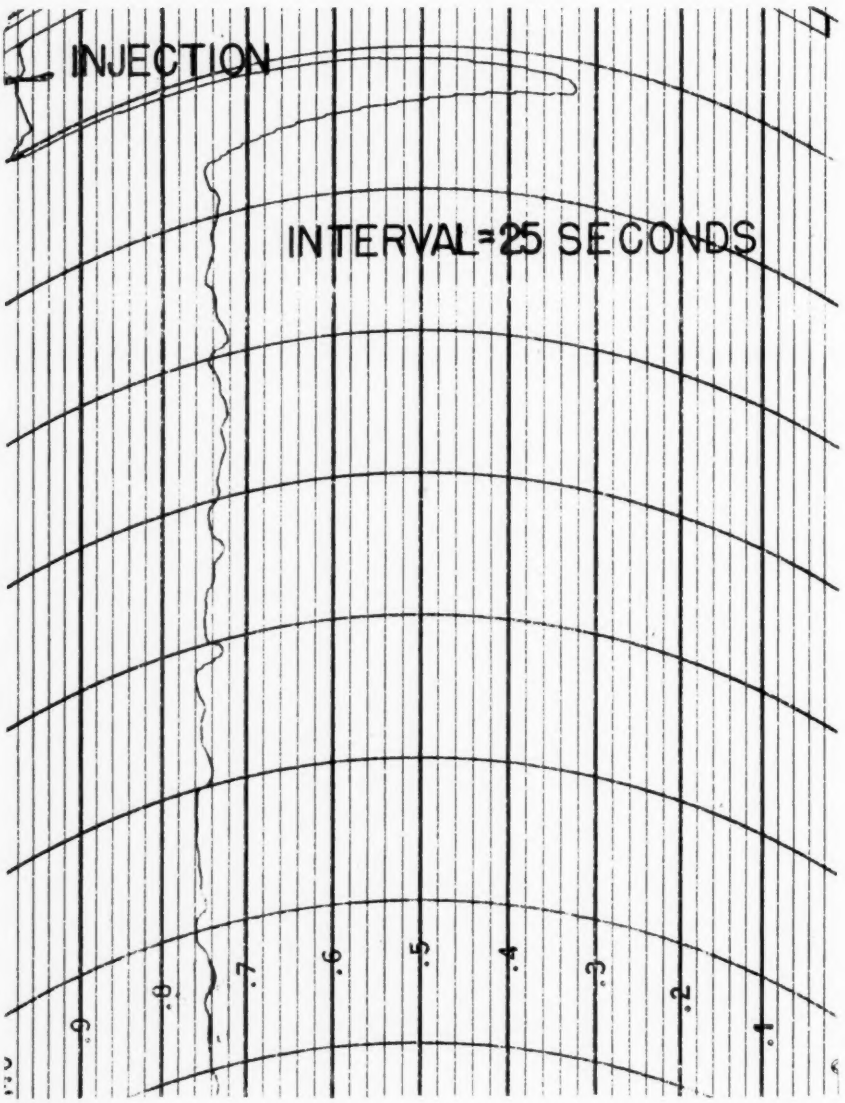

Fig. 3.—Graphic measurement of arm-to-ear circulation time using 20 mg. of T1824.

and the ear piece is applied to the right ear. There is no need for warning the patient about any sudden reaction. Illumination is next applied to the ear. The galvanometer deflection will decrease slightly within the next few minutes and then come to rest. This is due to increasing vasodilatation. Full-scale deflection is next obtained by means of a potentiometer placed in the output circuit. An 18 gauge needle is then inserted into the anticubital vein of the right arm and after a short pause approximately 3 c.c. of the dye* are

*0.5 per cent sterile solution of T1824, supplied by William Warner Company, New York, N. Y.

rapidly injected. A stop watch may be used to determine the time interval, to the nearest 0.5 second, between the moment of injection and the beginning of galvanometer deflection.

Some slight difficulty may be encountered in differentiating the beginning of galvanometer deflection from the steady fluctuation of the galvanometer due to respiration. The error in this regard is of no clinical significance since it is alway below 0.5 second. This difficulty is eliminated by using a graphic recorder. If a recording meter is used, the time interval may be determined by measuring the time base. (Fig. 3.) Circulation time measurements may be made to 0.1 second with this method. The determination may be repeated if desired.

OBSERVATIONS

A series of circulation time measurements using T1824 was made on a group of eighteen patients who did not exhibit any clinical evidence of congestive heart disease. In addition, repeat measurements were made within fifteen minutes using smaller quantities of dye (Table I). These values were then compared with those obtained with a standard clinical procedure using calcium gluconate.

Circulation time measurements with T1824 varied between 10.0 and 21.0 seconds, with an average of 14.2 seconds. In one instance both initial and repeat determinations revealed a circulation time of 21.0 seconds. This was observed in a 68-year-old emaciated white male patient. Two sets of determinations were obtained in Negro patients. In these instances the galvanometer deflection was reduced, but was still within adequate range to reveal a sharp end point. The greatest difference between repeat measurements was 2.0 seconds (Table I).

Measurement of the circulation time with calcium gluconate varied between 12.0 and 25.0 seconds, with an average of 18.0 seconds. In three instances the patients were confused in relation to the moment of heat sensation in the pharynx. The difference between the average circulation time obtained with T1824 and that obtained with calcium gluconate for each individual varied between 0.8 and 6.5 seconds.

DISCUSSION

Circulation time measurements obtained with T1824 are in general agreement with those reported for methods using various chemical substances. Goldberg,[1] using calcium gluconate, reported normal circulation times between 10 and 16 seconds. Bernstein and Simpkins[2] reported an average of 12.9 seconds for circulation times with magnesium sulfate. Magnesium sulfate has the added advantages of producing a sharper end point and being less toxic than calcium gluconate.[18, 19] Average circulation times in normal individuals of 20.8 seconds have been reported when papaverine was used,[4] 12.8 seconds with alpha-lobeline,[5] and 15.6 seconds with fluorescein.[11] Circulation times measured with calcium gluconate were always longer than those obtained with T1824 in this study.

TABLE I. EXPERIMENTAL PROCEDURE AND ARM-TO-EAR CIRCULATION TIME MEASUREMENTS USING T1824 AND CALCIUM GLUCONATE

PATIENT	APPARATUS	FILTER	INITIAL MEASUREMENT			REPEAT MEASUREMENT			DIFFERENCE (SEC.)	AVERAGE (SEC.)	Ca. GLUCONATE CIRCULATION TIME (SEC.)	DIFFERENCE BETWEEN Ca. GLUCONATE & DYE METHODS (SEC.)
			DYE INJECTED (MG.)	CIRCULATION TIME (SEC.)	DEFLECTION (CM.)	DYE INJECTED (MG.)	CIRCULATION TIME (SEC.)	DEFLECTION (CM.)				
A. S.	Oximeter	Standard	15	12.0	1.8							
			15	11.0	0.8							
			5	13.0	0.4							
M. R.	Oximeter	Standard	20	12.0	1.0							
			20	13.0	0.9							
			12	14.0	0.6							
A. R.	Oximeter	Standard	20	15.0	1.4							
J. R.	Oximeter	Standard	20	13.0	1.6	5	13.0	0.6		13.0		
P. W.	Photometer	Wratten*29	15	11.0	12.7	13	12.0	10.2	1.0	11.5		
F. H.	Photometer	Wratten 25	20	12.0	2.0	15	11.5	1.2	0.5	11.7	14.0	+2.3
L. D.	Photometer	Wratten 29	20	16.0	2.0	5	15.5	0.9	0.5	15.7	17.5	+1.8
			10	17.0	1.0	5	16.5	0.5	0.5	16.7		
J. B. (Negro)	Photometer	Wratten 29	20	17.0	0.7	15	17.5	0.8	0.5	17.2	19.0	+1.8
L. T.	Photometer	620	20	10.0	7.6	4	10.5	1.5	0.5	10.3	12.0 face 14.0 tongue	+3.8
W. M.	Photometer	620	20	11.5	4.1	5	12.0	2.9	0.5	11.7	14.0	+2.3
L. C.	Photometer	620	17	21.0	4.4	8	21.0	1.9		21.0	25.0	+4.0
R. McG.	Photometer	620	20	14.0	5.4	4	14.0	1.8		14.0	19.0	+5.0
H. S.	Photometer	620	20	14.0	5.8	4	14.5	1.5	0.5	14.2	18.0	+3.8
L. T. (Negro)	Photometer	620	20	10	3.6	4	12.0	0.7	2.0	11.0	16.0	+5.0
N. D.	Photometer	620	17	17.0	1.8	8	17.0	0.7		17.0	19.0	+2.0
H. B.	Photometer	620	20	13.0	6.1	4	14.0	0.6	1.0	13.5	20.0	+6.5
R. S.	Photometer	620	17	12.0	8.1	4	14.0	3.1	2.0	13.0	19.0	+6.0
T. B.	Photometer	620	20	17.0	10.4	4	18.0	2.1	1.0	17.5	21.0	+3.5
H. R.†	Photometer	620	26	15.0	9.5	3	15.2	2.0	0.2	15.1	21.0	+5.9
Average			18.2	14.2	5.7	6.6	14.7	2.0	0.7		18.3	

*Wratten gelatin filters obtained from Eastman Kodak Company, Rochester, N. Y.
†Circulation time measurement performed with Esterline-Angus graphic recording meter, Fig. 3.

The differences observed between repeat measurements with T1824 were 2.0 seconds, or less, in all cases. A difference of 2.0 seconds was observed in two instances, the remainder were within one second. Lilienfeld and Berliner[20] reported differences of 3 to 199 per cent in repeat determinations using alpha-lobeline, with failure to obtain any reaction in 20 per cent of their patients. With papaverine, differences in duplicate determinations have been as great as 3 seconds.[4]

SUMMARY

The azo dye T1824 may be used as an objective measure of circulation time. Inherent errors of subjective methods are eliminated and patients are not aware of any discomfort during the progress of the test. The test may be performed in individuals having deeply pigmented skins with only slight sacrifice of galvanometer deflection.

The use of monochromatic light and a high-sensitivity indicating apparatus permits detection of minute amounts of dye in the blood stream. Validity of circulation time measurements with T1824 is dependent upon the sensitivity and time constant of the amplifier. The amplifier described has a short time constant and high degree of sensitivity. Amplification is sufficient to produce galvanometer deflection as high as 10.4 centimeters. In addition, provisions are made for continuous recording of dye concentration in the blood stream.

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